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IMMOBILIZATION OF A FUNGAL CARBOXYMETHYLCELLULASES BY GEL ENTRAPMENT AND ITS IMPACT ON OPERATIONAL STABILITY

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ABSTRACT

Slightly acidic form of purified CMCases (13.97 μmg^{-1} protein) was immobilized in 13.6 % (w/v) acrylamide in the presence of cross linker Bisacrylamide which is 2 % of the monomer acrylamide, 0.02 ml TEMED and 0.01g ammonium per sulfate per ml was added to reaction mixture which initiated polymerization. 71.34 % protein was immobilized while 28.34 remain unbound. The polymerized gel containing CMCases was cut and sliced into 1×1 mm fragments. It is stored at 4° C. its operational and recycling activity was observed for consecutive four operations. Enzymes were heated in a solution two hours each times. Immobilized CMCases showed V_{max} (49 $\mu mol\ mg^{-1}\ mL^{-1}$), K_m (4.54 $mg\ mL^{-1}$), and specificity constant (V_{max}/K_m) 10.78 in its fourth operation and consecutive application.

Key-words: *Arachniotus citrinus*, immobilization, gel entrapment, operational stability.

INTRODUCTION

Enzymatic processes in industries are usually operated between 30-90°C at ordinary pressure. Enzymes are labile and thermally very sensitive. These are denatured at high temperature and lose their catalytic activity. Therefore, enzymes have very limited industrial application. The main problems of their high cost of production, difficulty in their recovery from reaction products for eventual recycling instability towards temperature, pH, storage and biodegradability. To overcome these problems, enzymes on affixing with some suitable carrier are made reuseable, repeatedly and conveniently separable. So enzymes are immobilized to meet the industrial challenges of adverse conditions of temperature, pH, storage and recycling. This reduces the cost of enzyme production and increases its industrial use. For this purpose, immobilization is carried out. Immobilization is an artificial and intentional containment, compartmentation, packing, inclusion or entrapment of enzymes in polymeric matrices or binding and affixing onto surface of a carrier due to physical and chemical interaction to restrict mobility, leakage and diffusion of enzymes and make it repeatedly re-usable (Buchholz and Kasche, 1997; Mosbach, 1971). The immobilization of enzyme has improved the stability towards storage, temperature, pH, denaturant as well as resistance to microbial attack and autolysis. Immobilization can be carried by different methods: Immobilization by adsorption is the linkage of enzymes with suitable hydrophilic solid surface. This is due to mild ionic interaction with solid support. Enzymes adhere firmly when mixed, stirred or incubated with suitable carrier at appropriate pH and temperature (Palmer, 1981). Immobilization by non-covalent interaction is very useful for non-aqueous system. It is a simple method in which chemical composition, surface and structure of enzymes remains unaltered. Enzymes simply attach to inert organic /inorganic or bio-polymers by Vander Waal's forces. Immobilization by ionic bonding provides slightly more specific way of enzymes attachment to carrier. Therefore, many ions-exchange resins as DEAE-Sephadex, CM cellulose, Dowex, Amberlite and DEAE cellulose are used (Naby *et al.,* 1999).

Immobilization by covalent bonding through functional groups provides more permanent linkage between enzyme and carrier. Enzymes are coupled with surface through activated matrix and bifunctional reagent. The functional groups which undergo covalent bonding with the carrier are sulphahydryl, hydroxyl, imidazol, amino and carboxyl. The coupling agents mostly used are trichlorotriazine, N-hydroxysuccinamide ester, Benzoquinone, glutaraldehyde, ester, CNBr, EDC and azides (Ulbrich and Schellenberger 1986,). Immobilization by entrapment, inclusion or encapsulation method involves the enzymes being retained within the membrane device such as hollow fiber, polymeric network, gel lattice or microcapsule. The immobilization by entrapment of enzyme in polymeric network is strongly effected by degree of polymerization and pore size which depend on the concentration of the monomer and cross linker (Naby *et al*., 1999, Palmer, 1981). Immobilization techniques are helpful to study the enzyme in their natural milieu (Mosbach 1971) because enzymes are entrapped in cellular proteinic net work, reticule or assemblage. So the practical approach to study the enzymes in a simulated natural environment is the

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immobilization by microencapsulation or gel entrapment (Wingard, 1972). The present study is to check the effect of immobilization on availability of enzymes for catalysis and operational stability.

MATERIALS AND METHODS

Immobilization of Carboxymethylcellulases

Polyacrylamide gel was prepared according to the partially modified method of Roy *et al*., (1984). The polymerization mixture contained 4ml of CMCases (75 mg enzyme powder ml^{-1}) in 50mM sodium acetate buffer pH, 5.0 (13.97µ mg^{-1} protein), 5ml of 13.6% (w/v) acrylamide and cross linker Bis-acrylamide which is 2% of the monomer (acrylamide) was added and vertimixed. N, N, N, N-tetramethyl ethylenediammine (TEMED) 0.02 ml and 0.01g of ammonium persulphate dissolved in 0.2ml of distilled water, were finally added to the above polymerization mixture as a catalyst. The total volume of the mixture was kept upto10ml. Finally the mixture was poured in the Dual Gel Caster of Mighty Small SE 245 Hoefer Scientific San Francisco. The whole process was carried out at 25°C. After polymerization the gel was cut and sliced into 1x1 mm fragments and washed several times with sodium acetate buffer (pH 5.0) to remove all unbound enzyme. The immobilized enzyme was kept at 4°C for further use and study. Proteins and CMCases activity was measured after each washing according to Bradford (1976) and Miller (1959).

Determination of Immobilized Proteins

The immobilized enzymatic protein was calculated by subtracting the amount of unbound protein from the protein initially added using Bradford method (Bradford, 1976).

Enzyme Assay

A fixed volume of 1.5 % (w/v) CMC sodium salt in 50 mM sodium acetate buffer pH 5 was warmed at 40°C for 5 minutes. Immobilized enzyme (150mg gel contains 33.46 µg protein) and soluble CMCase (2.175µg protein) were added separately in the warmed substrate and incubated at 40°C for one hour. The immobilized enzyme was immediately separated after micro centrifugation, 3 ml of DNS reagent was added in the reaction mixture. It was boiled in water bath for 10 minutes and cooled in ice bath before absorbance was taken at 550 nm (Ng and Zeikus, 1988). Enzyme activity was calculated.

$$\mu ml^{-1}min^{-1} = \frac{\Delta A_{550}}{\sum_m} \times \frac{V_t \times D_f}{V_e \times t}$$

Whereas
ΔA= absorbance; $\sum_m$ = Extinction coefficient; V_t = total volume of reaction mixture;
V_e = volume of enzyme; D_f = dilution factor; T = time in minute; µ = enzyme activity

Functional stability

The immobilized CMCases were repeatedly used to determine its V_{max}, K_m and V_{max}/K_m after 1st, 2nd, 3rd, and 4th successive continuous operations. Fixed amounts of immobilized CMCases were added to varied conc. of CMC i.e 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5% (w/v) and were incubated at 50°C for two hours. Kinetic constants V_{max}, K_m, and V_{max}/K_m were determined by Lineweaver-Burk double reciprocal plot by using Slide Write Computer Software. Immobilized enzyme was rinsed, washed and again used against the above mentioned conc. of the CMC substrate and again the whole process was repeated to determine kinetic constants. In this way operational stability in the light of decrease of V_{max}, increase of K_m, mass transfer and leakage of enzyme was determined.

RESULTS AND DISCUSSION

Immobilization of CMCases

Immobilization of CMCases was done to make it reusable. Immobilization of enzymes may be carried out by different methods such as adsorption, ionic or covalent bonding and entrapment etc. But entrapment within the network is the most suitable and widely applied immobilization method (Mosbach and Mosbach, 1966).
The results of immobilization of CMCases showed that 71.34% proteins were entrapped in the gel while other remained unbound and washed out (Table 1) Concentration of cross- linker plays an important role in protein immobilization. According to Naby *et al*. (1998), increasing the level of cross linking monomer (N, N-methylene bisacrylamide) from 2-5% resulted in a gradual decrease of enzyme activity and immobilization yield. This may be due to matrix porosity and development of diffusional resistance for both substrate and product. Similar results were reported for other immobilized enzymes by entrapment in polyacrylamide gel (Fatah *et al*., 1997).

Table 1. The rate of entrapment of proteins (CMCases) in polyacrylamide gels.

Cross linking concentration	Total protein added (μg)	Unbound protein (μg)	Immobilized protein (μg)	Immobilization Yield (%)
2%	2900	831	2069	71.34

Table 2. The Immobilization rate of CMCases in polyacrylamide gel.

CMCases	Units
a) Added	40.54
b) Theoretically immobilized (a-c)	28.59
c) Washing (unbound)	11.59
d) Actually immobilized	15.05
e) % Immobilization ratio (100 x d/b)	52.64

The activity corresponding theoretically to the amount of enzyme immobilized in the gel was calculated as the difference between enzyme activity of original enzyme and the activity lost in all washes after immobilization was 28.59 units (Table 2) The remaining activity in supernatant was proportional to the amount of enzyme originally added (40.54 units). The actual activity of the CMCases in entrapped gel was 15.05 units (Table 2).The difference (13.54 units) between the theoretical and measured activity of the immobilized enzyme indicated that entrapment process caused loss of activity compared to free enzyme. The amount of immobilized enzyme was not proportional to its activity. The theoretical activity may be affected by interactions between the substrate and the carrier or substrate and enzyme (diffusional accessibility phenomena). The large loading of enzyme could also result in overcrowding of the protein molecules. Similar results were reported by Busto *et al*. (1995), Shimizu and Ishihara (1987) and Vaidya *et al*. (1987).

Table 3. Functional stability of immobilized CMCases from *Arachniotus citrinus* at 50 ^{o}C.

Application	V_{max}	K_m	V_{max}/K_m
Ist	78.7	33.33	2.361
2nd	21.0	4.54	4.62
3rd	15.8	5.0	3.16
4th	49.0	4.54	10.79

Functional Stability

The immobilized CMCases remained entrapped in the gel and did not leak out even after four consecutively repeated applications. The enzyme was heated in the substrate solution for two hours each time. The specificity

constant value for all repeated cycles were gradually improved which showed that CMC was becoming more and more specific even after four repeated applications (Table 3, Fig. **1**). Due to repeated use and incubation for longer time, gel porosity has been increased and diffusional limitations to substrate and product imposed by gel network have been reduced. Immobilized CMCases exhibited Vmax (49 µmole /mg/min), K_m (4.54 mg ml^{-1}) and specificity constant (10.78) V_{max}/K_m (Table 3, Fig. **1)**. Woodward *et al*. (1993) reported that β-glucosidase was thermally stable at 40° C for several months. During this time it was used for a continuous process of cellobiose hydrolysis without any loss of efficiency. Naby *et al*., (1999) reported immobilized dextranase was active even after twelve continuous cycles of reuse. Immobilized enzyme columns of the fusion protein bound to cotton fibers exhibited stable β-glucosidase for at least ten days of continuous operations at 37°C (Ong *et al*., 1991). Furthermore immobilized β-glucosidase remained fully active during two weeks of continuous operation at 50°C (Le *et al*., 1994).

CONCLUSION

In this study it is concluded that 71.34 % protein were immobilized and activity lost in all washes after immobilization was 28.59 units. The activity of enzyme originally added in the form of supernatant was 40.54 units. The enzyme bound activity was 15.05 units and entrapment process caused a loss of 13.54 units. The amount of immobilized enzyme was not proportional to its activity. The loss of activity may be due to diffusional accessibility phenomena. Large loading of enzyme may also result in over crowding of protein molecules. However, repeated application and large incubation time has increased porosity and diffusional limitations to substrate and product imposed by gel network have been reduced. Immobilized CMCases exhibited V_{max} (49 µmol mg^{-1} mL^{-1}), K_m (4.54

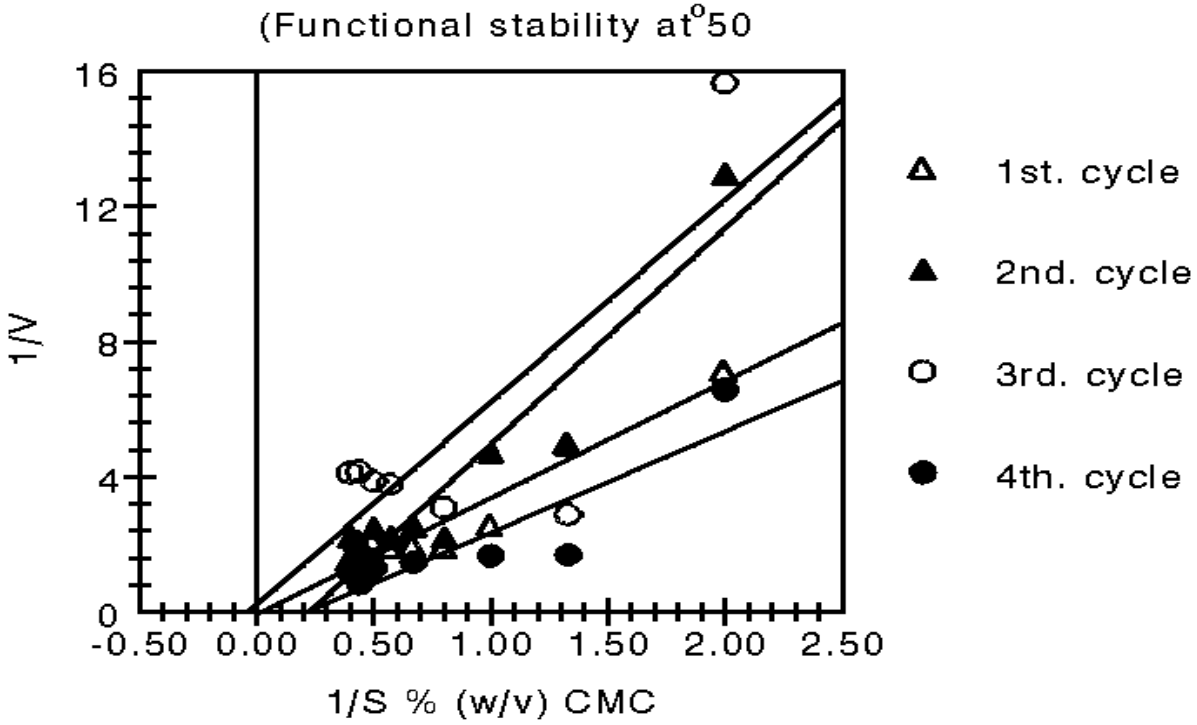

Fig. 1. Functinal / operational stability of CMCases in polyacrylamide gel.

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