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Inactivation thermodynamics and iso-kinetic profiling for evaluating operational suitability of milk clotting enzyme immobilized in composite polymer matrix

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ABSTRACT

Milk clotting enzyme (MCE) was immobilized in alginate-pectate interwoven gel with the yield of 73%. The encapsulated enzyme retained most of the protein load while soluble enzyme lost major proportion of activity after few hours. The immobilized enzyme showed high operational stability by retaining 40% activity even after 10 uses. The narrow optimal working pH of soluble enzyme changed to a broader range after encapsulation and a shift in optimum temperature from 45 to 50 °C was also recorded for encapsulated enzyme. Studies on isokinetic temperature showed that immobilized enzyme is more thermo-stable at higher temperature. Immobilization, therefore, not only improved the catalytic properties and stability but also its suitability in food processes like cheese preparation with reduced cost and time.

1. Introduction

Despite the wide spread research efforts in academics and industry, the number of applications in which biocatalysts have been employed remains modest. This may be attributed to several limiting factors like high cost, instability and non-availability of biocatalysts in pure form. It may further be compounded with sensitive nature of this macromolecule whose catalytic activity depends on its three dimensional structure and conformation [1]. Any permanent or temporary changes in its natural conformation caused by any chemical or physical agent affect its catalytic function. Therefore, it is highly essential to protect an enzyme to exploit its catalytic activity. In-spite of the remarkable catalytic properties of an enzyme, it has to be made robust before its implementation at an industrial scale [2].

Immobilization of enzymes on suitable supports has been observed to overcome these limitations. Immobilized enzymes have many advantages over free enzymes including easy separation of catalytic components, engineering design for continuous process, potential for greater efficiency in consecutive multi-step reactions, repeated use with sustainable half-life with less decay rate etc [3]. The enhanced operational stability of the immobilized biocatalysts has also been of great significance for their exploitation at commercial level [4]. Immobilization most often stabilizes the structure of enzymes thereby allowing their applications even under harsh conditions of temperature, pH and organic solvents.

Although immobilization method differs from enzyme to enzyme, from application to application and from support to support, depending on the peculiarities of specific application, yet the criteria for assessing the robustness of immobilized enzymes remain essentially the same i.e. high activity in a unit of mass or volume ($U\,g^{-1}$ or $U\,mL^{-1}$), high selectivity (to reduce side reactions), high stability (to reduce cost by effective reuse), economical (low cost contribution thus economically attractive) and innovative (for recognition as intellectual property) [4].

Enzymes can be immobilized on to a multitude of different carriers by entrapment, adsorption, ionic binding and covalent binding [4]. The basic idea behind enzyme immobilization is either to covalently attach or to entrap the protein in gel lattice which prevents it from leaching while allowing substrates, products and cofactors

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to permeate through [5]. The desirable characteristics of solid supports used for immobilization include large surface area, low cost, reusability and good chemical, mechanical and thermal stability. A number of supports like agar, alginate, porous glass beads, chitin, fiber, silica, polyacrylamide and polymers of polyvinyl alcohol with boric acid have been used in the recent past to immobilize either the whole cells or the enzymes [6–8].

Direct addition of enzyme to the cheese milk was not successful due to loss of enzymes in the whey, poor enzyme distribution, reduced yield and poor-quality of cheese. Incorporation of encapsulated enzyme eliminated the problems associated with direct enzyme. Amongst the various methods used, entrapment in alginate-pectin matrix is considered as the most preferable method. Hydrocolloids like alginates are copolymer of β-D-mannuronic acid and α-L-glucuronic acid linked by $1 \rightarrow 4$ glycosidic linkage whereas pectin is copolymer of galacturonic acid residues. Both forms hydro-gel in the presence of cations such as calcium, copper, zinc etc and can retain molecules greater than 5 kDa. Small sized enzyme molecules easily leak out from the gel so the gel strength has been increased using pectin as an admixture.

Although milk clotting enzymes (MCEs) have many applications in cheese industry and recently authors [9,10] have reported on their production and purification, yet their commercial exploitation can only be realized after immobilization. In the present investigation, attention hasbeen focused on encapsulation of MCE using biopolymers like alginate which has numerous applications in food processing, textile industry and pharmaceutical sector. Selection of alginate as a gel matrix for enzyme entrapment was based upon its compatibility, cheapness and sugar free gel formation and their reusability in process protocol of cheese preparation.

2. Materials and methods

2.1. Microorganisms and chemicals

Bacillus subtilis MTCC 10422 isolated from the soil of milk plant was used in this study. Ammonium sulfate, Sephadex G-100 and casein (*Hammerstein*) were products of Sisco Research Laboratory (Mumbai, India). Sodium alginate and LMP (low methoxyl pectin) were supplied by Fluka (Buch, Switzerland). Rennet from *Mucor miehei* type II was procured from Sigma Chemicals Co., USA. All other chemicals used were of analytical grade.

2.2. Cultivation and production

B. subtilis MTCC 10422 isolated from soil was maintained on modified yeast extract milk agar media (YEMA) supplemented with sucrose, at 4 °C by transfer onto a fresh medium after every 2–4 weeks. The enzyme was produced under liquid state fermentation (LSF) conditions in yeast extract milk media (pH 6.0, temperature 40 °C) [9]. The bacterial growth was allowed for 48 h of incubation. The cell free broth was separated through microfiltration. The obtained filtrate was centrifuged at 10,000*g* for 10 min at 4 °C. The clarified homogenate so obtained was further purified to homogeneity [10].

The starter culture used in cheese preparation (CH-149) procured from National Collection of Dairy Culture, ICAR-NDRI, India was activated by series of transfers from stock culture to sterilized skim milk. The working culture was propagated and maintained in sterile skim milk. The bulk culture was prepared a day prior to its use in cheese making.

2.3. Optimization of immobilization

The purified MCE was immobilized by entrapment within alginate-pectin beads. The effects of different concentrations of composite constituents for building the support material were investigated by varying the ratios to obtain stable alginate-MCE-pectin beads (alginate to pectin ratio from 1.8:0.2, 1.6:0.4 and 1.4:0.6) and cationic solution used was $CaCl_2$ (in the range of 0.1–0.5 M). The emulsion samples were hydrated 24 h before being stored until use at 4 °C to minimize bacterial growth. Afterwards the biopolymers dispersions were sterilized at 121 °C for 15 min, cooled to 4 °C. Enzyme formulations were diluted with 0.2 mM $CuCl_2$ for enhanced stabilization as reported earlier [11]. The biopolymer emulsion was mixed with enzyme aliquots, filled and ejected through the syringe into the $CaCl_2$ solution. The synthesis of biopolymer immobilization matrix in presence of enzyme is called perfect entrapment [12].The solution was gently stirred for 30 min to avoid deformation. Initially, enzyme beads of different sizes were obtained by using needles of variable gauges (G) but smaller beads were preferred for commercial application. The beads were collected using filter funnel and stored overnight in sterile 0.07% $CaCl_2$ solution at 4 °C for hardening. During entrapment, the ratio of biocatalyst-biopolymer solution to calcium chloride was kept 1:10 (*v*/*v*). The calcium chloride suspension was centrifuged at 3000*g* for 10 min to estimate unbound enzyme.

All polymers were prepared in Milli-Q water at higher concentrations than mentioned above so the desired concentrations could be obtained when mixed with the enzyme solution.

2.4. Milk clotting assay and protein estimation in various preparations

The procedure used was a slight modification of the method described earlier [13]. Five mL of the assay milk (10% skim milk and 0.01 M $CaCl_2 \cdot 2H_2O$ in distilled water) was taken in a test tube, contents brought to 37 °C in a constant temperature water bath and 0.5 mL of enzyme extract was added. Curd formation was observed while manually rotating the test tube from time to time so as to form a thin film on its inner surface. The end point was recorded when discrete particles were discernible. The procedure used for estimating proteolytic activity was a slight modification of the method described previously [14]. The amount of protein in different preparations was estimated by Lowry's method using bovine serum albumen as the standard protein [15].

2.5. Characterization of encapsulated MCE

2.5.1. Influence of reaction parameters

The effect of temperature and pH on entrapped MCE was evaluated in the temperature range 30–60 °C and pH range 4.0–9.0. The results of optimal pH determination were obtained by isothermal assaying the enzyme preparations at constant substrate (0.65%) and temperature (40 °C). The buffers of constant molarity (100 mM) were: acetate (pH 3.5–5.5), MES (pH 5.5–6.5) and Glycine–NaOH (pH 7.0–8.5). The pH stability of enzyme preparation was evaluated by pre-incubating at different pH values for 30 min at 40 °C. Initial activity was considered 100% and the residual activity was measured for next 24 h at regular intervals. Temperature optima was determined by assaying MCE for 10 min in pre-optimized buffers at different temperatures (30–60 °C). The pre-standardized optimal value of pH was used for thermal assays. Thermal stability was determined by incubating enzyme preparations at different temperatures for different durations. Enzyme aliquots were withdrawn regularly and the residual activity was calculated.

2.5.2. Thermodynamic determinants and thermal inactivation kinetics

The data obtained from the thermal stability profile was used to determine inactivation temperature as described earlier [16]. Afterwards, the inactivation temperature was used to analyze cor-