

Improved xylanase Characteristics upon enzyme entrapment Isolated from *Thermomyces lanuginosus* C9

Muhammad Irfan

University of Florida <https://orcid.org/0000-0002-1060-4436>

Jawairia Kiran

Quaid-i-Azam University

Salah Ud Din

Quaid-i-Azam University

Ameen ullah

Quaid-i-Azam University

Qurrat Ul Ain Rana

Quaid-i-Azam University

Malik Badshah

Quaid-i-Azam University

Fariha Hasan

Quaid-i-Azam University

Samiullah Khan

Quaid-i-Azam University

Aamer A. Shah (✉ alishah@qau.edu.pk)

Quaid-i-Azam University

Research Article

Keywords: Agar-agar Immobilization, Calcium alginate immobilization, *Thermomyces lanuginosus* C9, Enzyme kinetics, Thermostability, Thermogravimetric Analysis

Posted Date: August 3rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-744176/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Xylanases from microbial sources assume basic jobs in an assortment of industrial applications as a biocatalyst, and its applications generally require immobilization on supports to upgrade their stability. Enzyme immobilization is a thrilling decision to show signs of improved strength of enzymatic procedures. In this work, two sorts of polymeric backings (agar-agar and calcium alginate) are utilized to immobilize β -1,4-xylanase from *Thermomyces lanuginosus* C9 by entrapment, and afterward, biochemical properties of the entangled enzymes were performed. To create immobilized catalyst beads centralization of 4% agar while mix of sodium alginate 5% and calcium chloride 0.4 M was seen as ideal. Ideal reaction time for agar and calcium alginate immobilized protein increments from 10 to 25 and 30 min, separately. The incubation temperature expanded from 70°C to 75°C for agar however stayed unaltered for calcium alginate. The pH profile of free and immobilized xylanase was generally equal in both cases. Be that as it may, both the strategies changed the active boundaries of immobilized β -1,4-xylanase rather than free protein. High sub-atomic load of xylan limits dispersion which brings down the V_{max} estimation of immobilized protein while K_m value expanded. In contrast with agar-agar, protein immobilized inside calcium alginate display wide thermal stability and kept up 86.6% of its underlying activity at 80°C up to 150 min. Be that as it may, biotechnological portrayal demonstrated that the catalyst reusability was the most surprising discovery, predominantly of agar-agar immobilized xylanase, which held 31% activity after 7 cycles. These outcomes prove the biotechnical and monetary advantages of immobilization which help in an assortment of industrial applications.

Introduction

Xylan is a complex hetero-polysaccharide which is made out of xylose monomers these units are connected by β -1,4 linkages (10, 17, 18) Due to the broad interest of xylan hydrolytic items, for example, xylose, xylooligosaccharide, and xylobiose by microbial sources, the analysts focus on the improvement of various techniques for powerful degradation of xylan. One of the most significant techniques centered is the utilization of thermophilic biocatalysts which go about as solid and explicit cutting specialists (5). β -1,4 linkages can be cut by utilizing Endo- β -1,4-xylanase from thermophilic microbial sources (37, 16). After different biotechnological applications interest in this hydrolase has improved notably. Notwithstanding, with a few advantages, for industrial applications, the utilization of enzymes stays confined because of insignificant strength, catalyst loses its activity at different temperatures (19). This issue can be overwhelmed by utilizing thermozymes delivered from thermophiles which can be investigated in an expanded condition. Changed enzymes created by thermophiles from brutal ecological conditions can oppose diverse extraordinary states of being in the bioprocessing of various metabolites (12).

Biocatalysts delivered by thermophilic microorganisms of assorted sources have interested the biotechnological industries, and among them, immobilized thermozymes can diminish the expense of the bioprocess. With chemical and microbiological apparatuses, immobilization can be utilized to improve enzyme usage. Thermophilic catalysts uncover a fast hydrolytic rate, produce raised item yield, and are

less vulnerable to microbial contamination (38, 34). Then again, by utilizing diverse appropriate strategies the catalytic activities of a mesophilic catalyst can be improved. For the most part, after the development of the item, it is hard to isolate the protein from the reaction mixture because of the lower thermal and operational stability of the free enzyme. These confinements can be overwhelmed by immobilizing the protein inside various engineered and non-manufactured polymers to improve the dependability of the compound for steady metabolites creation (15, 11). Immobilization may give strength to the enzyme through multipoint connection, help to forestall the distance of catalyst, and can give positive conditions to the creation of various organic particles by enzyme-substrate reaction (24). Additionally, some other enzymatic properties, for example, enzymatic activity, specificity, protection from inhibitors, and so on can be improved by immobilization. Significant models in different bioprocesses are the choice of suitable immobilization method alongside the polymeric support. Among them, capture limits the enzyme to the organized network space and makes an unimportant effect on its catalytic properties For the ensnarement of various proteins (35) and cells different lattices, for example, agar-agar, polyacrylamide gel, and calcium alginate globules have been utilized. For the entanglement of organic macromolecules, agar-agar and calcium alginate are considered as a reasonable lattice due to their nontoxicity(20). Alginate is a characteristic polysaccharide made out of 1,4 connected β -D mannuronic and α -L-guluronic corrosive deposits. Alginate produces an insoluble gel-like structure (calcium alginate) within the sight of calcium particles which can withstand high temperature and is biocompatible with a considerable lot of the enzymes(6, 7).

Contemplating the expansive industrial utilization of thermostable endo- β -1,4-xylanase, the current examination was wanted to immobilize the mostly cleaned endo- β -1,4-xylanase. Protein was created from an indigenously isolated *Thermomyces lanuginosus* C9 and immobilized inside agar-agar framework and calcium alginate beads utilizing entrapment strategy. For free and immobilized enzymes distinctive reaction parameters were analyzed. The thermal and operational stability of immobilized enzymes was likewise researched to achieve the necessity of various industrial procedures. Additionally, inspected the Surface geology of gel globules with and without protein.

Material And Methods

Production of endo- β -1,4-xylanase

Thermomyces lanuginosus C9 was isolated from the underground aquifer of Garam Chashma Chitral Khyber Pakhtunkhwa, Pakistan. Confine was distinguished as dependent on Cultural, Morphological, biochemical, and 16s rRNA sequencing. Xylanase from *Thermomyces lanuginosus* C9 was delivered utilizing following medium (gL-1): xylan 0.5g; Peptone 8g; Glucose 8g; Yeast separate 0.03g; K_2HPO_4 8g; KH_2PO_4 2g; $CaCl_2$ 0.04g; $Na_3C_6H_5O_7$ 0.60g; NaCl 5g; $MgSO_4$ 0.05g with pH 7.0 was utilized for Xylanase creation and afterward hatched at 55°C for 24 hrs.

The cell-free filtrate was obtained by filtration of the content of each flask through Whatman filter paper number 1. The supernatant solutions, hereafter called crude extracts, were obtained from filtration

procedures use as xylan-degrading enzyme preparation. Precipitation of xylanase was performed at 70% saturated ammonium sulfate. The obtained precipitates were dissolved in phosphate buffer (100 mM, pH 7.0) and bring it into a dialysis membrane (pore size 10kDa) to be desalted against the same buffer at 4°C overnight. For entrapment within agar-agar matrix and calcium alginate beads, these dialyzed precipitates were further used

Immobilization of xylanase onto calcium alginate bead and within agar-agar matrix

Entrapment of dialyzed xylanase in agar-agar solution was accomplished by blending equivalent measures of xylanase in 1:1 proportion with agar-agar solution. Concentrated agar-agar solution was set up by vigorous shaking at 100°C. This solution was chill off at 55°C and consequently, the xylanase was varying cautiously before the gelling temperature. Cementing was accomplished in sanitized Petri plates. The cemented agar-agar gel containing xylanase was cut into little beads (5.0 mm by metallic stopper drill) and at last the immobilized enzyme beads were gauged 0.5g to be utilized for additional examination. Control was utilized as an Agar-Agar bead containing no enzyme.

For the synthesis of steady beads of calcium alginate, Sodium alginate (10.0–70.0 gL⁻¹) and calcium chloride (0.1–0.6 M) was utilized. Calcium alginate beads were set up for the lattice entrapment of xylanase. Incompletely purged compound was mixed in equivalent volume (1:1) of sodium alginate solution (50.0 gL⁻¹) arranged in sodium phosphate support (100 mM, pH-7.0). This blend was included dropwise into calcium chloride solution (0.4 M) with consistent mixing on the ice bath. Protein immobilized beads were shaped as insoluble beads. These beads were washed on various occasions with sodium phosphate buffer (100 mM, pH 7.0) to remove inexact bound xylanase. Alginate beads with and without immobilized xylanase were weighted and put away at 4°C for additional investigations. As control Calcium alginate beads without catalyst were utilized.

Enzyme Assay

To quantify the movement of free and immobilized catalyst Dinitro Salicylic Acid method (4) was utilized. All through the chemical measure, unrefined protein 100 µl was added to the reaction mixture containing 175 µl refined water, 125 µl buffer and 100 µl 5% substrate and afterward incubated at 60°C in a water bath for 10 min. A blank which contains all except the crude enzyme was additionally arranged. Promptly to each tube including blank 500 µl of DNS was included and was heated at 99°C in a water bath for 15 min. Be that as it may, for immobilized catalyst the method was adjusted. Premier 0.5g of immobilized beads were added to the reaction mixture containing 200 ml of 5% xylan and incubated at 60°C for 30 min. 1 ml from this reaction mixture was moved to another test tube containing 1ml DNS reagent. The rest of the convention was the same concerning the examination of free enzyme. At that point, measured the enzyme activity against blank by utilizing a UV-Visible Spectrophotometer at the frequency of 540 nm.

Enzyme unit: For xylanase unit of enzyme is known as “Under standard assay conditions the quantity of enzyme required to produce 1µmol/ml of reducing sugar per min”.

Effect of reaction time and temperature on the activity of the free and immobilized β -1,4-xylanase

To decide the impact of various reaction times the free and immobilized β -1,4-xylanase on agar-agar network and calcium alginate beads were incubated for various time stretches (5-50 min) under standard test conditions and then the residual activities were estimated. Different temperature ranges for example (30-80°C) and (30-90°C) were utilized to examine the impact of temperature on free and immobilized β -1, 4-xylanase on agar-agar framework and calcium alginate globules individually, and compound activity was determined in terms of residual activity

Effect of pH on the activity of free and immobilized β 1-4 xylanase

The impact of pH on the action of free and immobilized β -1,4-xylanase was determined by incubating with various pH supports extending from 3.0-11.0. Keeping a similar ionic quality (100 mM), the various buffers explored were: sodium acetic acid derivation (3.0-3.6), sodium phosphate (6.0-8.0), glycine NaOH (9.0-11.0).

Kinetic measurements

The kinetic measurements, for example, K_m and V_{max} of free and immobilized β -1,4-xylanase on agar-agar and calcium alginate polymers were resolved utilizing Lineweaver–Burk plot by performing the enzyme assay at various substrate concentrations running from 0.5-35 mg/ml.

Thermal and pH stability of the free and the immobilized β 1-4 xylanase

Thermal stability of free and immobilized β -1,4-xylanase was concentrated by incubating both at temperatures (30-80°C) for 10-150 min under standard assay condition and percent residual activity was determined.

The pH strength of free and immobilized β -1,4-xylanase (agar-agar and calcium alginate) was dictated by pre-incubating at various pH (3.0-11.0) for 10-150 min at the ideal temperature, and afterward, residual activity was determined.

Reusability of the immobilized β 1-4 xylanase

The catalytic activity of immobilized β -1,4-xylanase (agar-agar and calcium alginate) was expected to look at the operational stability of β -1,4-xylanase for steady processing. After each cycle Immobilized β -1,4-xylanase beads (agar-agar and calcium alginate dot) were washed with sodium phosphate buffer (100 mM;pH. 7.0) and re-exposed to the new substrate for the next group evaluation.

Scanning Electron Microscopy (SEM)

The surface morphology of beads with and without enzymes was examined through scanning electron microscopy (SEM). On a proper stub, the dried bead was mounted and afterward covered with carbon and falter Module in a vacuum evaporator. The covered samples were then seen under a scanning electron

microscope (MIRA3 TESCAN, institute of space technology, ISB) worked at 10 kV at the amplification of 5.0 kx, 10.0 kx, 25.0 kx.

Thermogravimetric Analysis (TGA)

To appraise the quality of polymer matrix and thermal stability thermogravimetric examination of agar-agar and calcium alginate beads with and without catalyst β -1,4-xylanase was executed dependent on percent weight reduction and deterioration. A thermal analysis instrument (METTLER TOLEDO, TGA 1 Stare framework) was utilized to perform TGA under nitrogen gas. In an alumina skillet around 3-7 mg of dried gel was gauged and heated the samples from 40°C to 1000°C at a heating pace of 10°C/min.

Results

Screening and identification of fungal strain for xylanases production

Fungal strain C9, which shaped clear zone around their colonies on xylan agar plates, was gotten for additional examinations. The isolate was affirmed as *Thermomyces lanuginosus* C9 with fractional 18S rRNA sequencing. The grouping was kept in GeneBank (Accession No. MK078054). The phylogenetic tree uncovered that *Thermomyces lanuginosus* C9 strain is firmly related (99.0%) with *Thermomyces lanuginosus* strain RMB (KF207598). (Data not provided).

Effect of concentration of polymeric material on immobilization of β -1,4-xylanase

Various concentrations of agar-agar (1-6%), as well as sodium alginate (1-7%) and calcium chloride (0.1-0.6 M), were utilized to entrap the β -1,4-xylanase inside the microenvironment of agar-agar and calcium alginate beads. A 4% (w/v) agar-agar focus was generally good for the capture of β -1,4-xylanase, giving a 62.2% entrapment yield (Fig. 1a). Correspondingly, it was discovered that the most extreme entrapment was accomplished when 5% sodium alginate and 0.4 M convergence of CaCl_2 , i.e., 8% and 20% separately (Fig. 1b,c). Immobilization proficiency diminished which may be because of the development of beads having a little pore size that makes prevention enter the substrate in calcium alginate beads and react with immobilized enzyme. Nonetheless, a low concentration of sodium alginate brought about the formation of soft and delicate beads with enormous pore size which brought about the expanded catalyst spillage during the washing step (23). In this way, an appropriate concentration of sodium alginate is important for the most extreme immobilization of protein.

Effect of reaction time on activity of free and immobilized β -1,4-xylanase

The impact of reaction time on the general action of free and captured β -1,4-xylanase was additionally concentrated by incubating entrapped β -1,4-xylanase with a substrate for various time (5.0-50.0 min). It was seen that agar-agar and calcium alginate immobilized β -1,4 indicated the most extreme hydrolytic action at 25 and 30 min separately when contrasted with a free compound which was accomplished at 10 min (Fig. 2).

Effect of temperature on the activity of free and immobilized xylanase

To watch the ideal temperature for maximum enzyme-substrate reaction-free and immobilized β -1,4-xylanase was incubated at various temperatures going from 30 to 90°C. The optimum temperature for the most extreme catalytic activity of agar-agar and calcium alginate immobilized β -1,4-xylanase was expanded from 70°C to 75°C as contrast with free enzyme (Fig. 3a). In this case, immobilized catalyst demonstrated 93% relative activity at 80°C when contrasted with free protein which was roughly 87%.

Effect of pH on the activity of free and immobilized β -1,4-xylanase

To examine the most extreme enzyme-substrate reaction of free and immobilized β -1,4-xylanase thought about the wide scope of pH (3.0-11.0). No noticeable impact of entrapment saw on the pH go for the action of β -1,4-xylanase. Notwithstanding, over the pH range 9–10 activity was somewhat upgraded (Fig. 3b).

Thermal stability of free and immobilized β -1,4-xylanase:

For the utilization of enzymes in harsh industrial procedures, thermal steadiness is one of the key variables. Because of a short-lived lifetime in industrial settings the utilization of soluble enzyme is limited which presents a significant common-sense issue for their use in the modern procedure. After entrapment, the steadiness of the compound is accepted to increment concerning time and temperature. The thermal stability of immobilized β -1,4-xylanase as contrast with the free compound is expanded by entrapment at different temperatures extending from 30°C-90°C. The agar-agar captured β -1,4-xylanase lost its activity much more slow rate at various temperatures and was seen that the stability of immobilized β -1,4-xylanase as contrast with free catalyst was expanded from 70% to 87.2% at 80°C and 17 % to 44.5% at 90°C. In like manner, the strength of calcium alginate entangled β -1,4-xylanase was expanded from 85.4% to 91.4% at 70°C and 71.5 % to 86.6% at 80°C when contrasted with free catalyst (Fig. 4).

Repeated use of matrix entrapment of endo- β -1,4-xylanase

Reusability is one of the major outstanding components of the entrapped enzyme inside the polymer. Agar-agar and calcium alginate entangled enzyme reusing effectiveness was inspected up to seven rehashed cycles and it was seen that under operational conditions the reactant productivity of network captured β -1,4-xylanase was tremendous and indicated over 50% and 40% action after the second and fourth cycle, separately. While calcium alginate beads held just 34% and 16% activity after fifth and 6th cycles (Fig. 5)

Enzyme kinetics of free and immobilized β -1,4-xylanase

The affinity of an enzyme toward its substrate is spoken to as Kinetic constant (K_m) though the higher enzymatic reaction rate is shown as maximum reaction rate (V_{max}). For free and immobilized protein K_m

and V_{max} values were determined by utilizing the Lineweaver-Burk plot, by estimating compound action at various substrate focuses running from 0.5-35 mg ml⁻¹, while keeping the pH and temperature constant. It was strangely noticed that matrix entanglement somewhat increased K_m value though decreased the V_{max} value. For agar-agar captured protein K_m value expanded from 4.19408mgml⁻¹ (free catalyst) to 5.32mgml⁻¹ (immobilized) while V_{max} value was diminished from 235.78 $\mu\text{molmg}^{-1}\text{min}^{-1}$ (allowed) to 50.25 $\mu\text{molmg}^{-1}\text{min}^{-1}$ (immobilized) individually (Fig 6a,b). While for calcium alginate immobilized xylanase the K_m value of immobilized compound was diminished to 2.77mgml⁻¹ as contrasted with free enzyme 4.19408mgml⁻¹ which may prompt quicker reaction rate and V_{max} value was diminished from 235.78 $\mu\text{molmg}^{-1}\text{min}^{-1}$ (allowed) to 9.56 $\mu\text{molmg}^{-1}\text{min}^{-1}$ (immobilized) (Fig. 6a,c).

Surface Morphology of enzyme immobilized beads

Polymer gel surface morphology when entrapment of β -1,4-xylanase was explored by utilizing scanning electron microscopy. And micrographs were seen at different amplification scales. The micrograph result showed displayed a clear variation between the surface geography of beads with and without immobilized β -1,4-xylanase. Polymer gel without catalyst show void pores and moderately smooth surface when amplified (Fig. 7a,c,e) (Fig. 8a,c,e), Whereas, after enzyme entrapment, the pores are appeared to be occupied by the enzyme crystal aggregates on the matrix surface in case of both agar-agar (Fig. 7b,d,f)) and calcium alginate beads (Fig. 8b,d,f)

Thermogravimetric Analysis (TGA) of enzyme immobilized Beads

Thermal stability of beads examined by thermogravimetric examination, by deciding rate weight reduction of beads with and without immobilized enzyme (agar-agar and Ca-Alginate). Consequently, exposed the beads with and without enzymes to the temperature range of 40-1000°C. Contrasted with the free catalyst the immobilized compound portrayed substantially more thermal stability. It is obvious from the figure (Fig. 9a) that the weight reduction for agar-agar globules with and without enzyme in a temperature range of 40-1000°C is about 58.1% and 68% individually While calcium alginate entrapped beads represent a weight reduction of 52% as contrast with the beads without enzyme which shows a weight reduction of 78%. (Fig. 9b)

Discussion

Immobilization is a strong strategy which not just allows simple recuperation of product and enzyme from reaction mixture yet, in addition, improves the thermal and operational stability of an enzyme. To accomplish the ideal entrapment yield impact of various concentrations of agar-agar (1–6%) as well as sodium alginate (1–7%) and calcium chloride (0.1–0.6 M) was optimized. The penetrability of immobilized enzyme beads is modified by the concentration of a matrix. It was discovered that the most extreme entrapment was accomplished at 4.0% agar-agar concentration and 5% sodium alginate and 0.4M calcium chloride concentration. Despite the way that higher concentrations of polymer favor enzyme entrapment, relative activity is decreased because of reducing entrance of the substrate to the active site of the protein transcendently high sub-atomic weight substrates (3). Then again, low

concentrations of polymer produce fragile gels not able to hold on to the enzyme. Thus, lower ensnarement yield is accomplished as the enzyme leak out during progressive washing. However, gels with bigger pore sizes permit dispersion of the substrate to the active site of the enzyme (21). According to past reports, Agar-agar concentration of 3% yielded the most noteworthy immobilization yield of laccase and protease (79.65 and 85%, individually) (3, 32). Higher agar-agar concentrations 4%, were required for viable immobilization of manganese peroxidase and lipase (3, 1). Various concentrations of sodium alginate and calcium chloride have been accounted for by different examination bunches for the entrapment of pectinase (27), protease (2), and Glucose oxidase.

Impact of reaction time on the action of free and agar-agar and calcium alginate immobilized β -1,4-xylanase was considered and it was noticed that the greatest xylan hydrolysis displayed by a free enzyme inside 10 min of reaction time while after immobilization the expansion in reaction time is seen up to 25 and 30 min. The reaction time of entrapped β -1,4-xylanase was expanded which maybe because of the time required by the substrate to diffuse into the unpredictable globule structure and comparable outcomes were accounted for in the event of immobilized pectinase utilizing the same support (28) and dextranase immobilization in calcium alginate beads where reaction time was expanded from 15.0 to 60.0 min (33).

The ideal temperature for the greatest catalysis of free and immobilized β -1,4-xylanase has been observed indicated that the immobilized catalyst is more stable than the free enzyme. In the two cases, the difference in ideal temperature from 70°C to 75°C indicated that immobilized enzymes required higher initiation vitality when contrasted with the free protein and as a result cause variety in the conformational adaptability of the enzyme inside the gel network (9). However, at high-temperature continuous decrease in percent relative activity was observed. As per the past exploration it is seen that the ideal temperature for immobilized cicer α -galactosidase was changed from 50 to 70°C while utilizing chitosan and amberlite as supports. This might be because of an impediment in the addition of kinetic energy because of limitation of free movement, the ideal temperature of entangled β -1,4-xylanase expanded.

The results for perfect pH show no immense effect on pH maxima. For the activity of an entrapped enzyme changes in the pH range depends upon the nature of the polymer and the properties of the compound. Generally, free sorts of enzymes are more affected by changes in pH than trapped proteins by buffering effect of the catch polymers. As showed by past reports, xylanase and dextranase when immobilized inside magnetic latex and calcium alginate beads no alteration in pH optima was observed of course α -amylase entrapment using agar-agar support, most extraordinary development was practiced at pH 7.0 when diverged from the free enzyme which exhibited activity at pH 5.5 (25)

The thermal stability of free and agar-agar and calcium alginate immobilized xylanase was likewise investigated by keeping them at different temperatures going from 30°C to 90°C for various periods. Improved thermal stability of an enzyme was noted when immobilized when contrasted with free protein. The rigidity of catalysts is expanded by polymer capture along these lines keeping it from denaturation at higher temperature and dragging out the existence of an enzyme. It was accounted for that different

variable including a number of bonds framed among enzyme and polymer, the nature of the bonds, the constraint level of an enzyme inside support, and the environmental conditions of a catalyst may influence the strength of immobilized protein.

The most significant parameter of immobilized compounds for industrial application is their rehashed use. In this way, the operational steadiness of xylanase captured in agar-agar and calcium alginate gel was surveyed by reusing the immobilized protein for seven cycles. It was seen that when the quantity of cycles expands the enzymatic activity of captured protein logically diminishes, which may be a result of the denaturation or filtering of the enzyme during over-the-top washing of entangled catalyst inside the agar-agar gel. The current outcomes are marginally higher than immobilized pectinase utilizing the same support while lower than stabilization of pectinase through a multipoint connection on enacted agar-agar gel (22). The operational stability of proteins has been accounted for increment by immobilization and recommended to be exploited on large scale. Alginate captured lipase was dynamic after 10 cycles with no loss in activity. In any case, 40 to 50% loss in activities of xylanase and lipase entrapped on κ -carrageenan and calcium alginate beads have additionally been noted (31).

Kinetic parameters of free and immobilized β -1,4-xylanase were likewise contemplated and discovered that the K_m value expanded and V_{max} value diminished for immobilized protein when contrasted with free enzyme yet if there should arise an occurrence of calcium alginate immobilized enzyme it was noticed that the K_m value diminished to 2.77mgml⁻¹ as contrasted with free catalyst 4.19408mg ml⁻¹ this might be because of the contrary charges on substrate and protein. It was accounted for that the K_m value for ficin immobilized on CM-cellulose azide diminished by 90% contrasted with that of free chemical. This abatement can be ascribed to partition effect because of which electrostatic powers between the matrix and substrate, because of which the concentration of substrate increments in the microenvironment of immobilized protein. As per past investigations increment in K_m value and lessening in V_{max} value were noted for catalysts immobilized in different polymers (13). Be that as it may, when xylanase was immobilized on polyalanine K_m and V_{max} values diminished (8). Though a portion of the examinations additionally detailed the expansion in K_m and V_{max} values (26). Distinctive sort of assays, polymers, and xylan utilized because of which change in the kinetic constant of xylan activity is consistently observed (29).

SEM was utilized to contemplate the morphology of beads with and without enzymes at different magnification scales. The contrast between the morphology of beads with and without compounds is shown by the micrograph results. The smooth surface can be seen when beads without protein are magnified though after entrapment the outside of the matrix is appeared to be occupied by enzyme aggregates as revealed if there should be an occurrence of pectinase immobilized on the same matrix. Similarly, Morphological changes on the outside of immobilized laccase were likewise detailed where TiO₂-montmorillonite complex was utilized as polymer (36)

TGA was utilized to research the thermal stability of agar-agar and calcium alginate beads with and without protein. Consequently, the globules were exposed to a temperature range of 4-1000°C. The

thermogram for both free and immobilized protein demonstrated a steady weight reduction. At first, at a temperature range of 40 to 200°C weight reduction is seen which might be because of the loss of truly adsorbed water. Extra weight reduction saw when the temperature is expanded up to 800°C which may be because of loss of natural moieties utilized for immobilization. At that point, a continuous decay was observed lastly the xylanase immobilized beads lost 58% (agar) and 52% (Ca-alginate) weight when contrasted with their partners having no catalyst, which lost 68% (agar) and 78% (ca-alginate) weight at 1000°C. This might be because of successful cross-connecting of the polymer matrix in light of dispersion of calcium into the alginate network during preparation. Different useful functional groups in the alginate-polymer structure edifices with calcium particles and the multifaceted nature of the process have been accounted for by other researchers (14). Additionally for protease immobilized MNPs the weight reduction saw to be about 7.86% somewhere in the range of 150 and 450°C which affirms the protease binding on MNPs (30).

Conclusion

All in all, the β -1,4-xylanase from *Thermomyces lanuginosus* C9 is thermophilic xylanase with maximal activity at 70°C and pH 7. β -1,4-xylanase was ensnared in agar-agar network and calcium alginate beads utilizing a straightforward and quick procedure with magnificent entanglement yield and no loss in relative activity. Entangled β -1,4-xylanase held its thermophilic properties, and had more noteworthy thermal stability than its free, soluble structure. Ensnared β -1,4-xylanase had incredible reusing capacity, demonstrated over 40% activity after the fourth cycle if there should arise an occurrence of agar-agar lattice While Ca-Alginate globule held 16% activity after the 6th cycle. The extraordinary properties of β -1,4-xylanase alongside high ensnarement yield, high relative activity in entangled structure, and reusability make it striking for different industrial zones.

Declarations

Acknowledgements

This study was financially supported by Higher Education Commission of Pakistan.

Conflict of interest

No conflict of interest is associated with this work

Ethics approval

Not applicable

Funding

The work was supported by Higher Education Commission Islamabad Pakistan.

Availability of data and material

NA

Code availability

NA

References

1. Adham, N. Z., Ahmed, H. M., & Naim, N. (2010). Immobilization and stability of lipase from *Mucor racemosus* NRRL 3631. *Journal of microbiology and biotechnology*, 20, 332–339
2. Anwar, A., Qader, S. A. U., Raiz, A., Iqbal, S., & Azhar, A. (2009). Calcium alginate: a support material for immobilization of proteases from newly isolated strain of *Bacillus subtilis* KIBGE-HAS. *World Applied Sciences Journal*, 7, 1281–1286
3. Asgher, M., Noreen, S., & Bilal, M. (2017). Enhancement of catalytic, reusability, and long-term stability features of *Trametes versicolor* IBL-04 laccase immobilized on different polymers. *International journal of biological macromolecules*, 95, 54–62
4. Bernfeld, P. (1955). In " *Methods in Enzymology* ".(Eds. SP Colowick and NO Kaplan.) Vol. New York: J. Academic Press
5. Bhalla, A., Bansal, N., Kumar, S., Bischoff, K. M., & Sani, R. K. (2013). Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresource technology*, 128, 751–759
6. Blandino, A., Macias, M., & Cantero, D. (1999). Formation of calcium alginate gel capsules: influence of sodium alginate and CaCl_2 concentration on gelation kinetics. *Journal of bioscience and bioengineering*, 88, 686–689
7. Blandino, A., Macías, M., & Cantero, D. (2000). Glucose oxidase release from calcium alginate gel capsules. *Enzyme and microbial technology*, 27, 319–324
8. Cakmakci, E., Danis, O., Demir, S., Mulazim, Y., & Kahraman, M. V. (2013). Alpha-amylase immobilization on epoxy containing thiol-ene photocurable materials. *J. Microbiol. Biotechnol*, 23, 205–210
9. Chang, M. Y., & Juang, R. S. (2005). Activities, stabilities, and reaction kinetics of three free and chitosan–clay composite immobilized enzymes. *Enzyme and Microbial Technology*, 36, 75–82
10. Collins, T., Gerday, C., & Feller, G. (2005). Xylanases, xylanase families and extremophilic xylanases. *FEMS microbiology reviews*, 29, 3–23.
11. Cowan, D. A., & Fernandez-Lafuente, R. (2011). Enhancing the functional properties of thermophilic enzymes by chemical modification and immobilization. *Enzyme and Microbial Technology*, 49, 326–346

12. Elleuche, S., Schroeder, C., Sahm, K., & Antranikian, G. (2014). Extremozymes—biocatalysts with unique properties from extremophilic microorganisms. *Current opinion in biotechnology*, 29, 116–123
13. Esawy, M. A., Gamal, A. A., Kamel, Z., Ismail, A. M. S., & Abdel-Fattah, A. F. (2013). Evaluation of free and immobilized *Aspergillus niger* NRC1ami pectinase applicable in industrial processes. *Carbohydrate polymers*, 92, 1463–1469
14. Fontes, G. C., Calado, V. M. A., Rossi, A. M., & Rocha-Leão, M. H. M. d(2013). Characterization of antibiotic-loaded alginate-OSA starch microbeads produced by ionotropic pregelation. *BioMed research international*, **2013**.
15. Guzik, U., Hupert-Kocurek, K., & Wojcieszynska, D. (2014). Immobilization as a strategy for improving enzyme properties-application to oxidoreductases. *Molecules*, 19, 8995–9018
16. Irfan, M., Gonzalez, C. F., Raza, S., Rafiq, M., Hasan, F., Khan, S., & Shah, A. A. (2018). Improvement in thermostability of xylanase from *Geobacillus thermodenitrificans* C5 by site directed mutagenesis. *Enzyme and Microbial Technology*, 111, 38–47
17. Irfan, M., Guler, H., Belduz, A., Shah, A., & Canakci, S. (2016). Cloning, purification and characterization of a cellulase-free xylanase from *Geobacillus thermodenitrificans* AK53. *Applied Biochemistry and Microbiology*, 52, 277–286
18. Irfan, M., Guler, H. I., Shah, A. A., Sal, F. A., Inan, K., & Belduz, A. O. (2019). Cloning, purification and characterization of halotolerant xylanase from *Geobacillus thermodenitrificans* C5. *Journal of Microbiology, Biotechnology and Food Sciences*, **2019**, 523–529.
19. Irfan, M., Kiran, J., Ayubi, S., Ullah, A., Rana, Q. U. A., Khan, S. ... Shah, A. A. (2020). *Immobilization of β -1, 4-xylanase isolated from Bacillus licheniformis S3*. *Journal of Basic Microbiology*
20. Kar, S., Mandal, A., Mohapatra, P. K. D., Samanta, S., Pati, B. R., & Mondal, K. C. (2008). Production of xylanase by immobilized *Trichoderma reesei* SAF3 in Ca-alginate beads. *Journal of industrial microbiology & biotechnology*, 35, 245–249
21. Kutcherlapati, S. R., Yeole, N., & Jana, T. (2016). Urease immobilized polymer hydrogel: Long-term stability and enhancement of enzymatic activity. *Journal of colloid and interface science*, 463, 164–172
22. Li, T., Li, S., Wang, N., & Tain, L. (2008). Immobilization and stabilization of pectinase by multipoint attachment onto an activated agar-gel support. *Food chemistry*, 109, 703–708
23. Longo, M. A., Novella, I. S., García, L. A., & Díaz, M. (1992). Diffusion of proteases in calcium alginate beads. *Enzyme and microbial technology*, 14, 586–590
24. Mateo, C., Grazu, V., Palomo, J. M., Lopez-Gallego, F., Fernandez-Lafuente, R., & Guisan, J. M. (2007). Immobilization of enzymes on heterofunctional epoxy supports. *Nature Protocols*, 2, 1022
25. Pal, A., & Khanum, F. (2010). Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. *Bioresour Technol*, 101, 7563–7569
26. Pal, A., & Khanum, F. (2011). Covalent immobilization of xylanase on glutaraldehyde activated alginate beads using response surface methodology: characterization of immobilized enzyme.

27. Rehman, H. U., Aman, A., Silipo, A., Qader, S. A. U., Molinaro, A., & Ansari, A. (2013). Degradation of complex carbohydrate: immobilization of pectinase from *Bacillus licheniformis* KIBGE-IB21 using calcium alginate as a support. *Food chemistry*, 139, 1081–1086
28. Rehman, H. U., Aman, A., Zohra, R. R., & Qader, S. A. U. (2014). Immobilization of pectin degrading enzyme from *Bacillus licheniformis* KIBGE IB-21 using agar-agar as a support. *Carbohydrate Polymers*, 102, 622–626
29. Romo-Sánchez, S., Camacho, C., Ramirez, H. L., & Arévalo-Villena, M. (2014). Immobilization of commercial cellulase and xylanase by different methods using two polymeric supports. *Advances in Bioscience and Biotechnology*, 5, 517
30. Sahu, A., Badhe, P. S., Adivarekar, R., Ladole, M. R., & Pandit, A. B. (2016). Synthesis of glycinamides using protease immobilized magnetic nanoparticles. *Biotechnology Reports*, 12, 13–25
31. Sardar, M., Roy, I. I., & Gupta, M. N. (2000). Simultaneous purification and immobilization of *Aspergillus niger* xylanase on the reversibly soluble polymer Eudragit(TM) L-100. *Enzyme Microb Technol*, 27, 672–679
32. Sattar, H., Aman, A., & Qader, S. A. U. (2018). Agar-agar immobilization: An alternative approach for the entrapment of protease to improve the catalytic efficiency, thermal stability and recycling efficiency. *International journal of biological macromolecules*, 111, 917–922
33. Ul, Q. S., Aman, A., Syed, N., Bano, S., & Azhar, A. (2007). Characterization of dextransucrase immobilized on calcium alginate beads from *Leuconostoc mesenteroides* PCSIR-4. *The Italian journal of biochemistry*, 56, 158
34. Viikari, L., Alapuranen, M., Puranen, T., Vehmaanperä, J., & Siika-Aho, M. (2007). *Biofuels* (108 vol.). Berlin: Springer
35. Walia, A., Guleria, S., Mehta, P., Chauhan, A., & Parkash, J. (2017). Microbial xylanases and their industrial application in pulp and paper biobleaching: a review. *3 Biotech*, 7, 1–12
36. Wang, Q., Cui, J., Li, G., Zhang, J., Li, D., Huang, F., & Wei, Q. (2014). Laccase immobilized on a PAN/adsorbents composite nanofibrous membrane for catechol treatment by a biocatalysis/adsorption process. *Molecules*, 19, 3376–3388
37. Wang, Y., Feng, S., Zhan, T., Huang, Z., Wu, G., & Liu, Z. (2013). Improving catalytic efficiency of endo- β -1, 4-xylanase from *Geobacillus stearothermophilus* by directed evolution and H179 saturation mutagenesis. *Journal of biotechnology*, 168, 341–347
38. Yeoman, C. J., Han, Y., Dodd, D., Schroeder, C. M., Mackie, R. I., & Cann, I. K. (2010). in *Advances in applied microbiology*, vol. 70, Elsevier, pp.1–55.

Figures

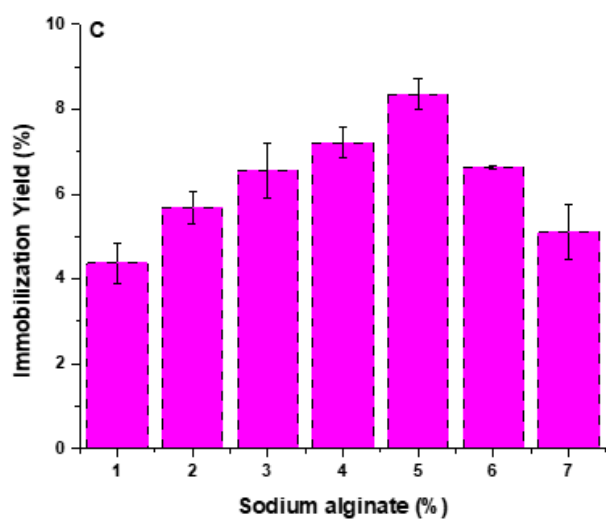
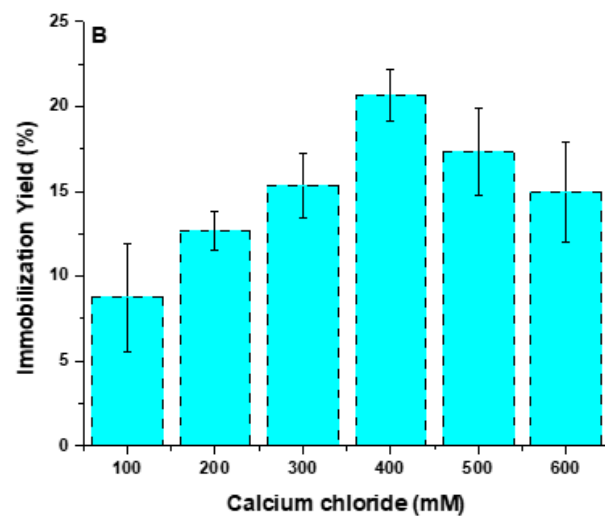
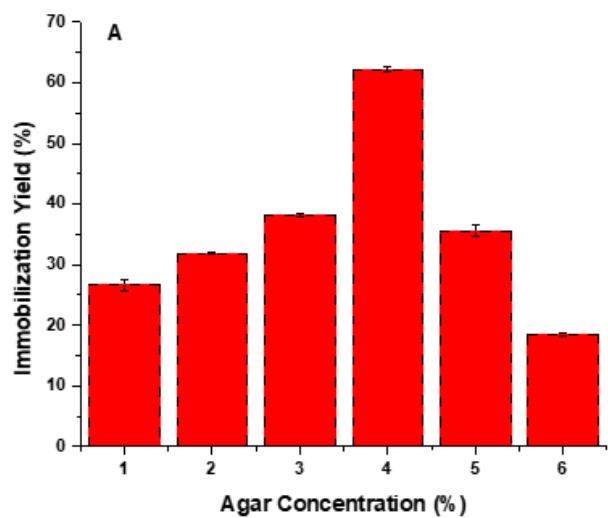


Figure 1

Effect of different concentrations of agar-agar (a) sodium alginate (b) and calcium chloride (c) on the immobilization of xylanase from C9

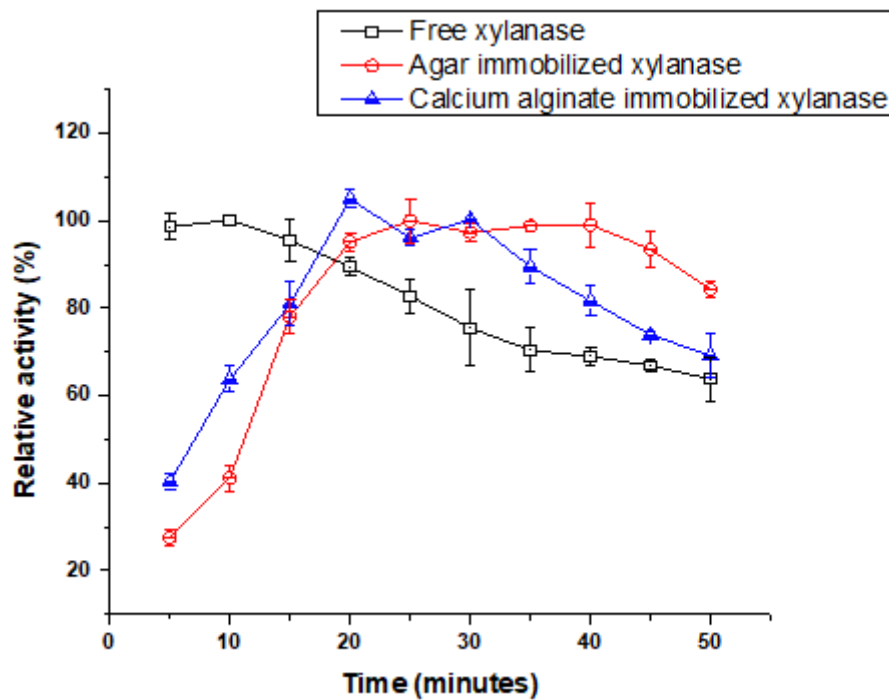


Figure 2

Effect of reaction time on the activity of free and agar-agar immobilized and calcium alginate immobilized xylanase from C9

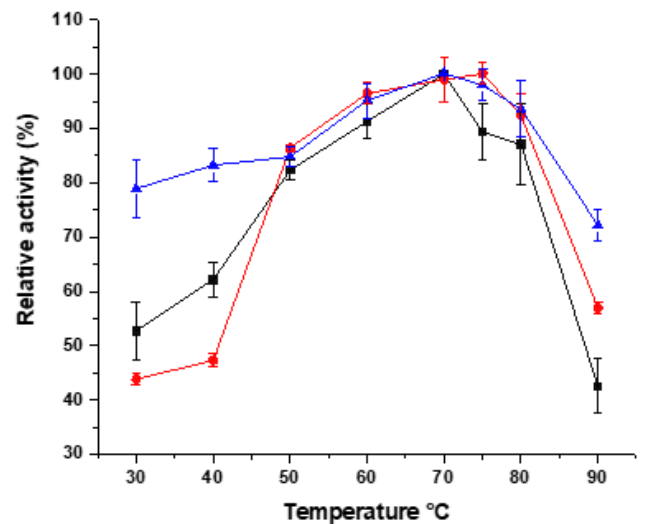
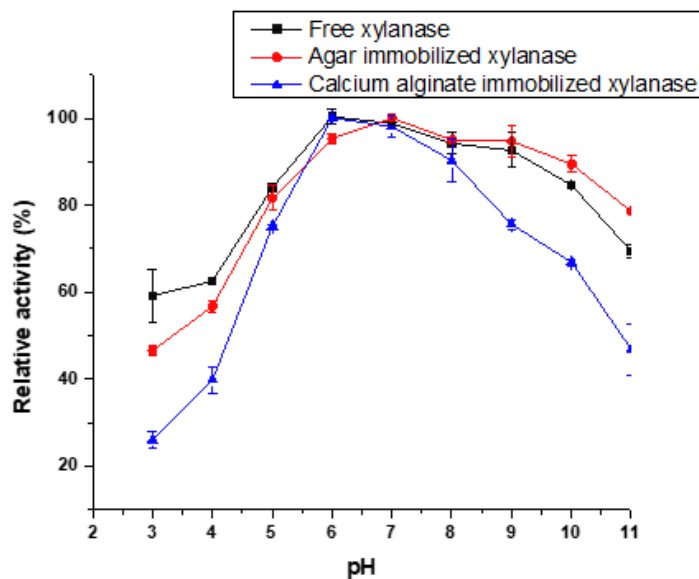


Figure 3

Effect of different temperatures (a) and pH (b) on the activity of free and agar-agar immobilized and calcium alginate immobilized xylanase from C9

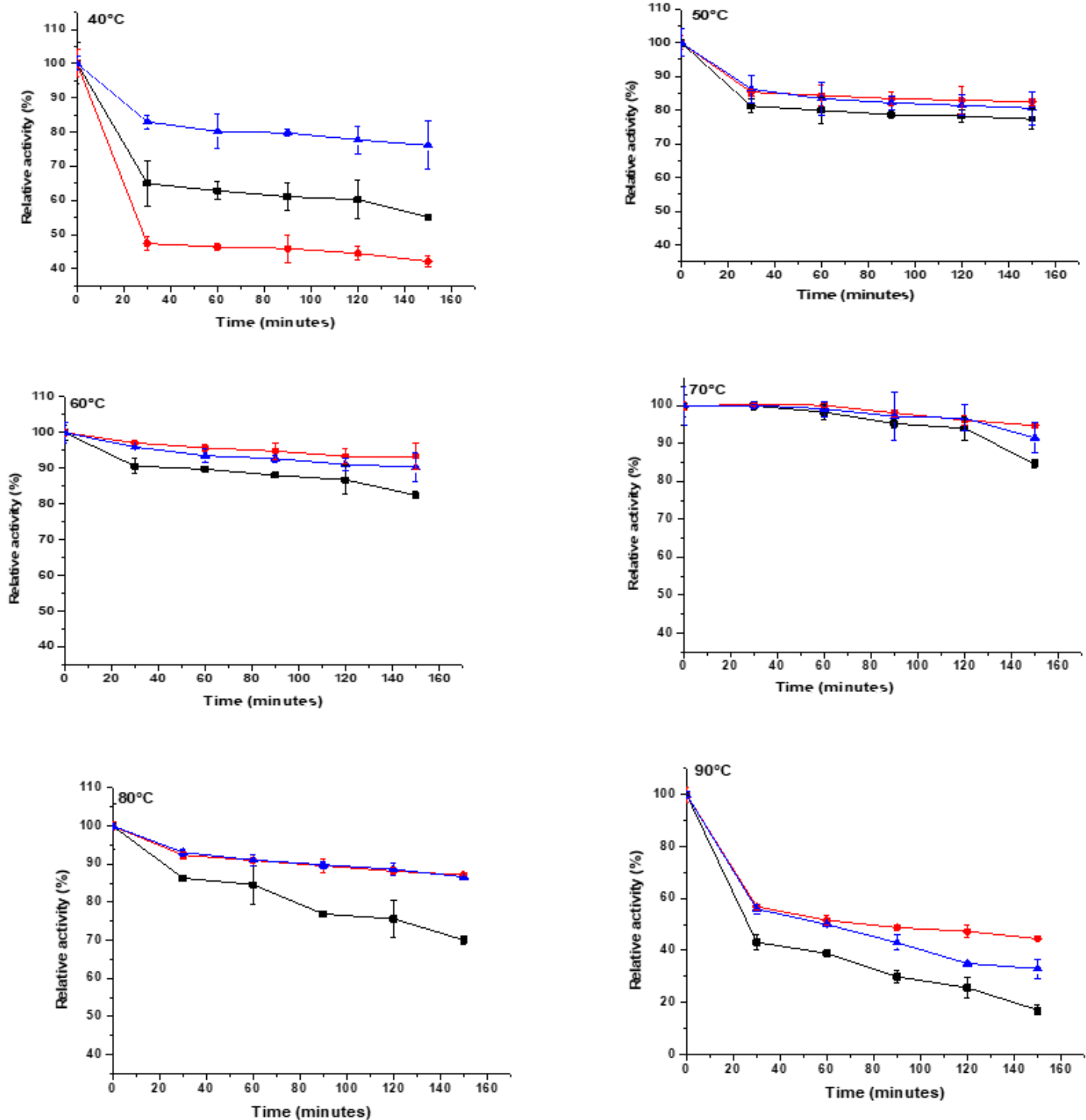


Figure 4

Effect of different temperatures on the stability of free and agar-agar immobilized and calcium alginate immobilized xylanase from C9 (40°C, 50°C, 55°C, 60°C, 70°C, and 80°C).

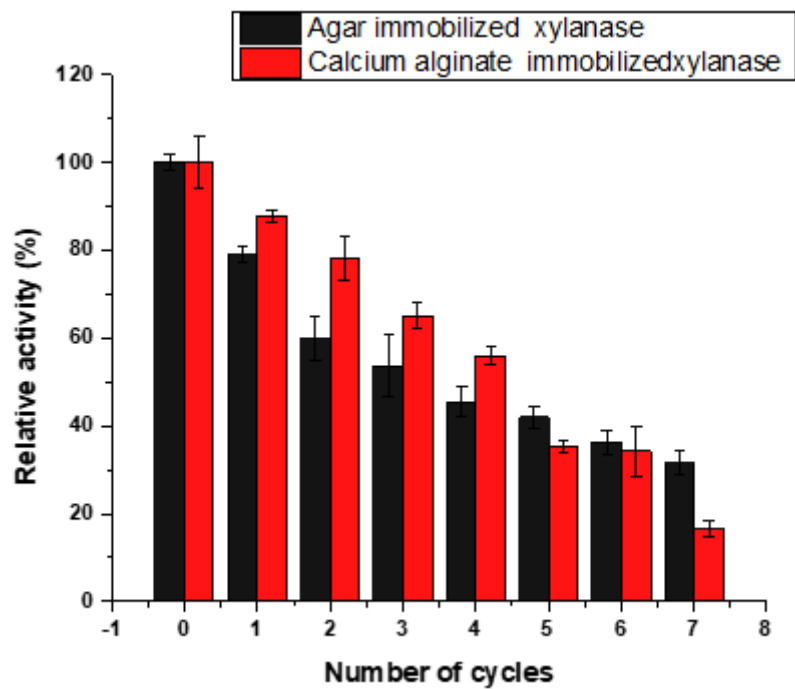


Figure 5

Reusability of agar-agar immobilized and calcium alginate immobilized xylanase from C9

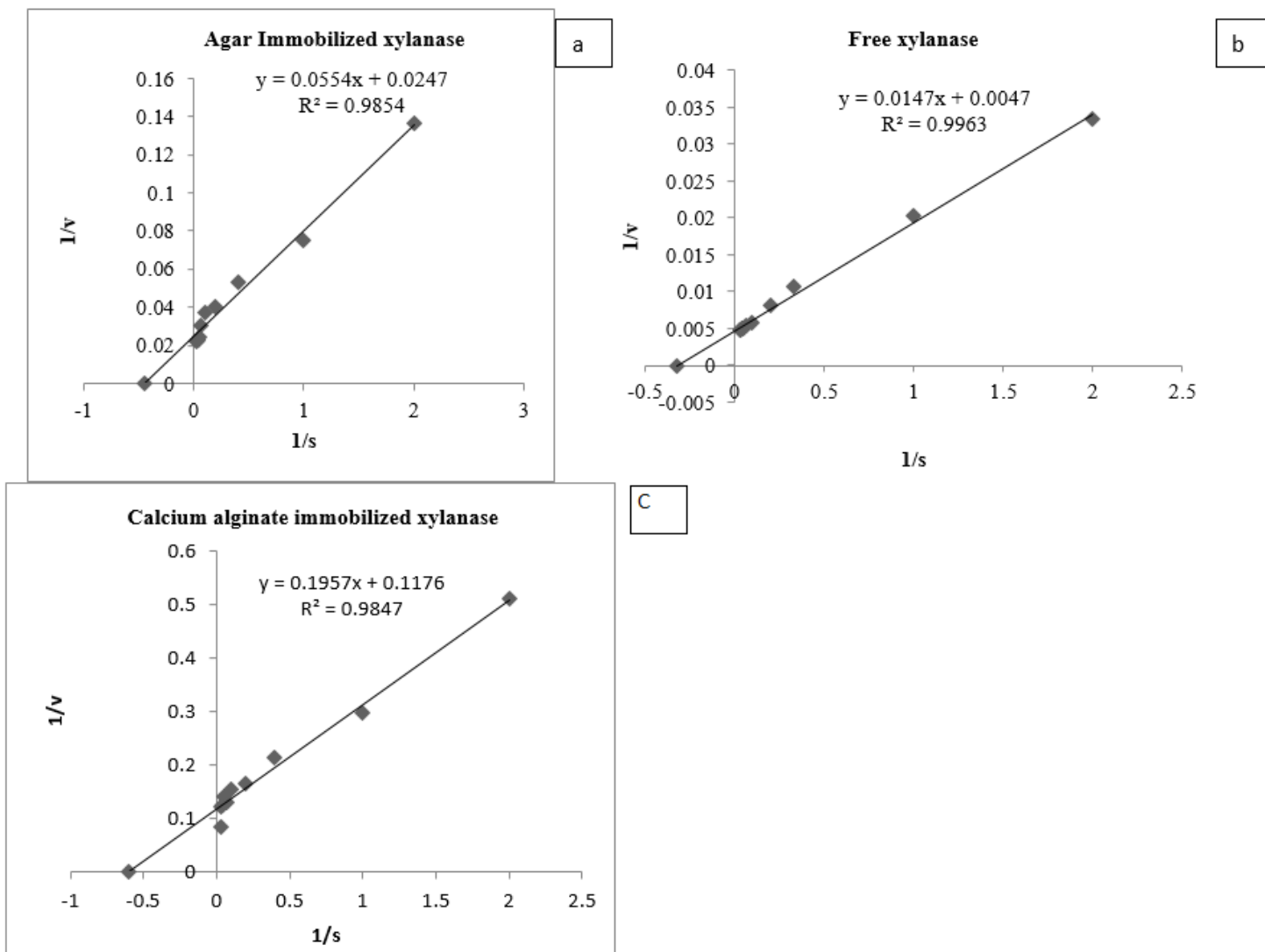
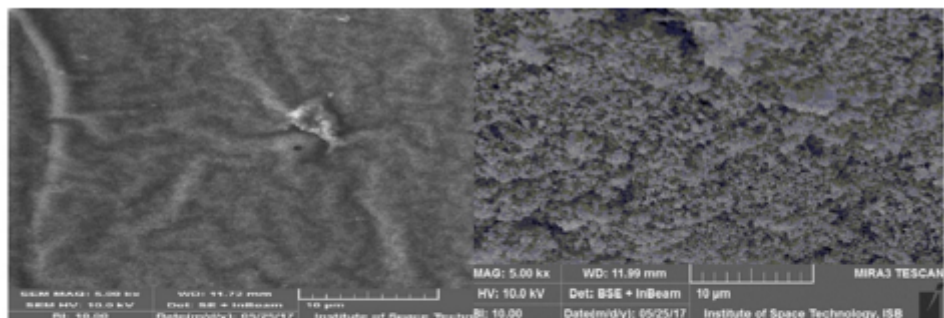


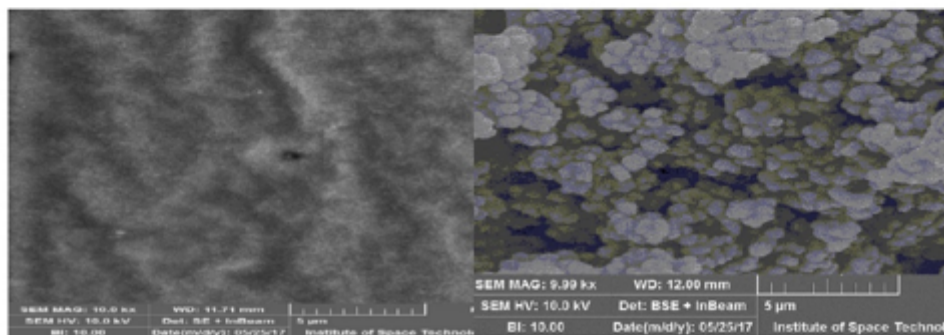
Figure 6

Lineweaver–Burk plot of free and immobilized xylanase from C9 (enzyme kinetics module of Origin pro 9 software was used to fit the initial velocity data into Lineweaver–Burk, and Michaelis–Menten constant equations). Free enzyme (a) agar-agar immobilized enzyme(b) Calcium alginate immobilized enzyme (c)



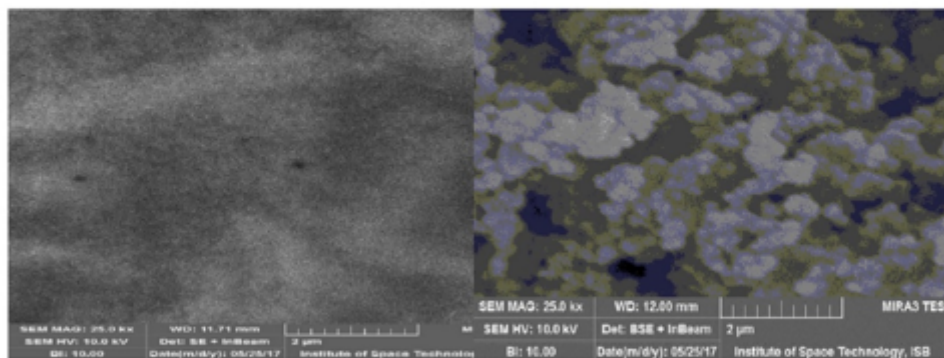
a

b



c

d

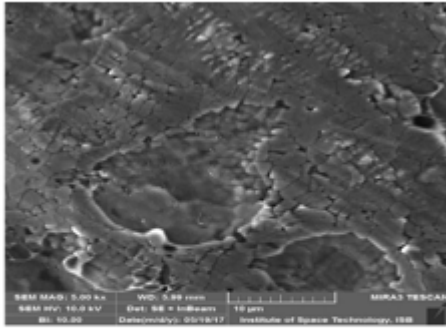


e

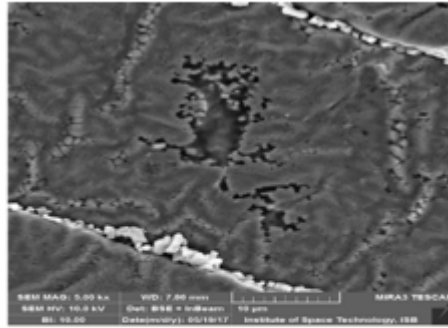
f

Figure 7

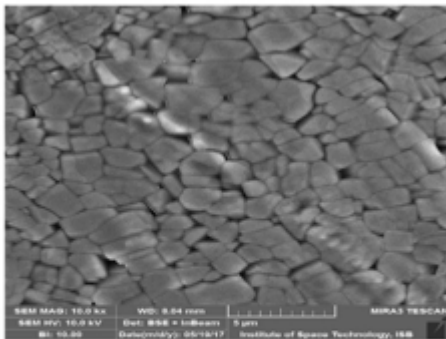
SEM of Agar-Agar matrix before (a,c,e) and after (b,d,f) immobilization of β -1,4-xylanase from C9 strain at different magnifications (5,00-25,00x)



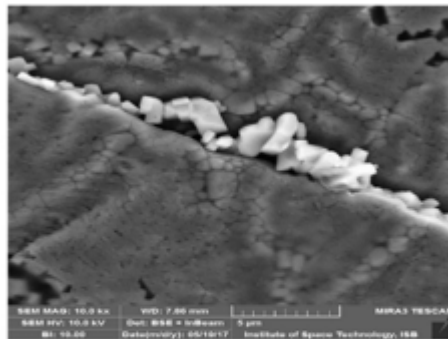
a



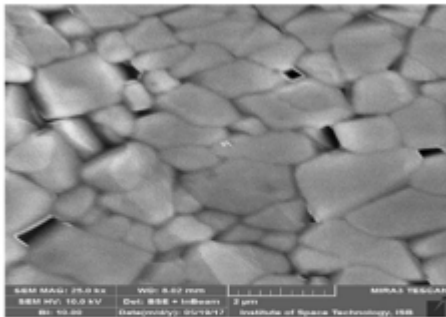
b



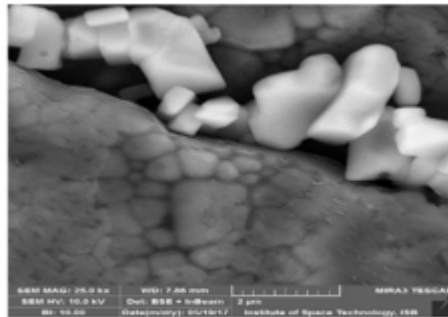
c



d



e



f

Figure 8

SEM of calcium alginate beads before (a,c,e) and after (b,d,f) immobilization of β -1,4-xylanase from C9 strain at different magnifications (5,00-25,00x)

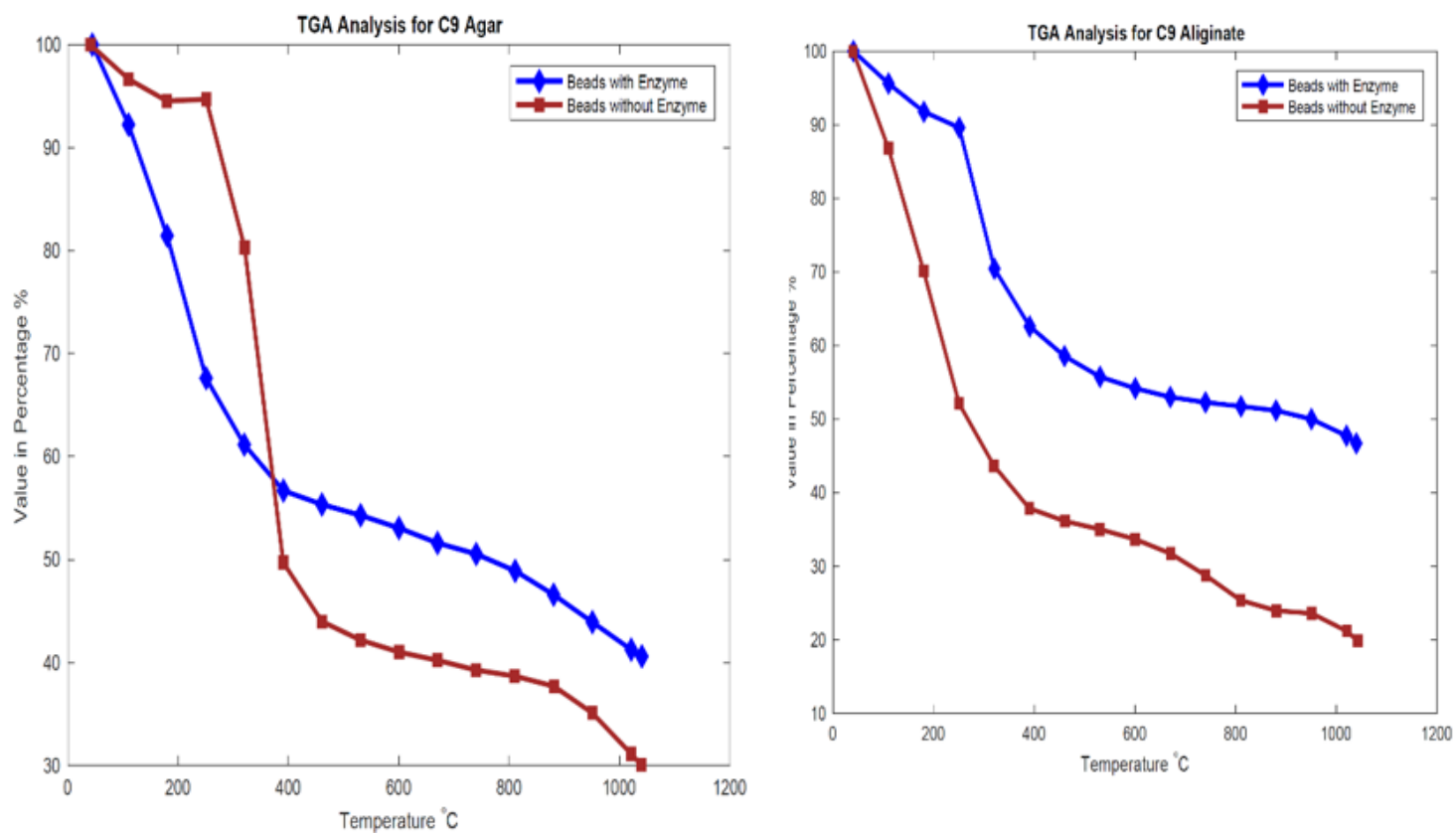


Figure 9

TGA thermograph of agar-agar (a) and Ca-alginate (b) beads with and without β -1,4-xylanase from C9