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Article

Three-phase partitioning and immobilization of *Bacillus methylotrophicus* Y37 cellulase on organo-bentonite and its kinetic and thermodynamic properties

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Abstract

In this study, for the first time *Bacillus methylotrophicus* Y37 cellulase was purified and recovered in a single step by three-phase partitioning (TPP). The optimal purification parameters for TPP were 40% ammonium sulfate saturation (m/v) with a 1.0:1.0 (v/v) ratio of crude extract:t-butanol, which gave 5.8-fold purification with 155% recovery of cellulase. Non-covalent immobilization of the partitioned cellulase was performed using bentonite as a support material. The activity observed in the 20th experiment was 100%. The optimal pH values and temperatures determined for the free enzyme and the immobilized enzyme were 5.0 and 6.0 and 45°C and 50°C, respectively. The Arrhenius activation energy (E_a) of the immobilized enzyme was lower than that of the free enzyme, whereas the Michaelis–Menten constant (K_m) and maximum velocity (V_m) of the immobilized enzyme increased. The turnover number (k_{cat}) and the catalytic performance (k_{cat}/K_m) demonstrated the improved catalytic properties of the immobilized enzyme compared to the free enzyme. Immobilization of cellulase is thermodynamically preferred.

Keywords: bentonite, cellulase, immobilization, kinetic, thermodynamics, three-phase partitioning

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Enzymes are natural biocatalysts. They are of benefit in industrial applications because they participate in environmentally friendly processes. The advantages of using enzymes are substrate specificity, the absence of undesirable side reactions, the mild conditions required for the reactions and non-contaminated product generation. Enzyme-based reactions usually have lower waste-treatment costs, which render it possible to construct and control the facilities using enzymatic reactions at much lower capital and energy costs (Hasan *et al.*, 2006; Patel *et al.*, 2019). Cellulase is a multicomponent enzyme consisting of three different enzymes (endocellulase, cellobiohydrolase and β-glucosidase), which together catalyse the hydrolysis of cellulose, generating soluble sugars. Cellulase has become one of the most important catalysts due to its wide range of industrial applications in the fields of food bioconversion, agriculture, pulp and paper, and textiles. It is particularly crucial in the bio-refinery industry, where it is utilized for catalysing cellulose into sugars.

Purification of this enzyme by operating various routine purification methods such as salting out and various chromatographic techniques is a costly and time-consuming procedure (Duman & Kaya, 2013b). Three-phase partitioning (TPP) is a bioseparation technique that can be used directly on crude suspensions and includes the principles of salting out, isoionic precipitation and cosolvent precipitation. It is a simple and low-cost purification technique and has been used to purify proteins, enzymes and inhibitors over recent decades (Duman & Kaya, 2013a). In TPP, ammonium sulfate at a particular level of saturation is used to precipitate the protein and t-butanol is added to make a three-layer phase and to remove lipids, phenolic compounds and some detergents (Rao *et al.*, 1998).

Most biological processes occur in the presence of biocatalysts. The acquisition of biocatalysts is expensive; thus, the overall economy of their use depends on their recovery and stability. The production costs of the enzyme constitute 50% of the total cost of the hydrolysis; hence, industrial applications of hydrolysis may be expensive. In order to achieve efficient recovery and stability of biocatalysts, immobilization has emerged as a system that provides suitable results in terms of high product yield in favourable conditions and allows for the reuse of the biocatalysts (Rao *et al.*, 1998). The speed and efficiency of the immobilization process depend on the type of carrier (support material), method of immobilization, concentration, pH, temperature and reaction time. Strong ionic, hydrophilic or hydrophobic and/or hydrogen bond interactions between the enzyme and the carrier affect the stability of the enzyme, as these strong interactions lead to irreversible adsorption of the enzyme on the carrier, resulting in a loss of enzyme activity. Such strong interactions may also cause conformational changes in the tertiary structure of the enzyme, which again lead to a loss in enzyme activity. These effects may be particularly notable in the case of multiple interactions on

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the surface of solid carriers (Worsfold, 1995). A variety of support materials, especially porous materials, are available for industrial applications, such as alginate, collagen, chitosan, agar–agarose, polyacrylamide, polyanhydrides and clays. The support material must be chosen on the basis of the production type, cost, product specificity, biocompatibility and usability (Naik *et al.*, 2016; Bilal *et al.*, 2018). The general expectations from an immobilization process are as follows: to provide a balanced bio-hybrid; to provide the necessary conditions in which the biomolecules do not become denatured during the process; to allow sufficient contact between the enzyme and the substrate; and finally to allow the estimation of the quantity of biomolecules required for the process (Vitola *et al.*, 2017).

Several techniques are available for the immobilization of enzymes. Selecting the most appropriate enzyme requires consideration of the result to be achieved. The major steps involved in the immobilization process are: (1) linking to the carrier, which may be achieved through covalent bonding, ionic bonding, physical adsorption and bio-specific binding; (2) cross-linking; and (3) encapsulation (Rao *et al.*, 1998).

Bentonites consist mainly of smectites, such as montmorillonite, beidellite, nontronite, saponite and hectorite, which have the same crystal structure but different chemical compositions. Smectites are 2:1 layered phyllosilicates, consisting of one octahedral sheet between two tetrahedral sheets that are bonded by oxygen bridges. Bentonites also contain non-clay mineral impurities. Some of these impurities can be removed by decantation and acid leaching. Smectites and thus bentonites have variable swelling capacities in water. Their specific surface area may be enlarged by acid activation at certain temperatures and times. In addition, smectites may intercalate various chemical species, including biomolecules, into their interlayer spaces (Grim, 1988; Alemdar, 2001; Naik *et al.*, 2016). Clays are reliable, environmentally friendly and cost-effective materials for carrying enzymes, so bentonite has been used as a suitable matrix for the immobilization of enzymes (Karakuş *et al.*, 2008; Dong *et al.*, 2012).

The TPP leads to proteins of great flexibility; increases in this conformational flexibility yield greater catalytic activity of the protein (Duman & Kaya, 2013b). On the other hand, although immobilization has become a useful technique because it improves reuse, it has some problems due to its structural rigidity and activity (Tavano *et al.*, 2018). In the present study, two methods known to increase and decrease the flexibility of enzymes were used. The cellulase enzyme derived from *Bacillus methylotrophicus* Y37 was partially purified by TPP and immobilized using bentonite. Bentonite was used for the process because it is inexpensive and widely available. The immobilization process was optimized and was also investigated in terms of kinetic and thermodynamic parameters.

Materials and methods

Materials

Cellulase (E.C.3.2.1.4) was obtained from *B. methylotrophicus* Y37 and was isolated from soil in a previous study (Duman *et al.*, 2016). Bentonite was obtained from Eczacıbaşı/Esan (Tuzla-İstanbul). Sodium carboxymethyl cellulose (CMC; molecular weight 90,000) and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma Aldrich (USA). Triton X-100, bovine serum albumin (BSA; 99%), disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium carbonate, sodium bicarbonate and calcium chloride glutaraldehyde were supplied by Merck AG (Germany). Coomassie Brilliant Blue G-250 potassium sodium tartrate and ammonia solution ($NH_3.H_2O$) were procured from Fluka AG (Switzerland). Hexadecyltrimethyl ammonium bromide (HDMAB; 98%) was purchased from Eastman Chemical Ltd (USA). All reagents were of analytical grade and were used without additional purification. All aqueous preparations were carried out in distilled water. All other chemicals and solvents used were purchased from one of the following: Eastman Chemical Ltd, Fluka AG, Riedel AG (Germany) and Merck AG, and were of analytical grade.

Three-phase partitioning

Effect of t-butanol on the partitioning of cellulose

Three-phase partitioning was carried out according to Duman & Kaya (2013a), with minor modifications. First, the effect of t-butanol on the crude extract was studied. The crude extract (5 mL) was saturated with 20% (w/v) ammonium sulfate at 25°C and vortexed gently for 2 min to dissolve the salt, followed by the addition of various ratios (v/v) (1.0:0.5, 1.0:1.0, 1.0:1.5, 1.0:2.0) of t-butanol to the crude extract. The mixture was vortexed gently and then allowed to stand for 1 h at 25°C. Then the mixture was centrifuged at 7000 rpm for 5 min at 4°C to form the three phases. The upper organic phase, the middle interfacial precipitate and the lower aqueous phase were separated carefully. The interfacial precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7). The lower aqueous layer and the interfacial phase were analysed for cellulase activity and total protein content. The interfacial phase containing cellulase was collected.

Effect of ammonium sulfate on the partitioning of cellulose

The effects of the salt concentrations (20, 30, 40 and 50%) on the crude enzyme in the TPP at a constant crude extract:t-butanol ratio (1.0:1.0) were also investigated. All of the experiments were performed in triplicate and the differences in the readings were <±5%. The data were expressed as means ± standard errors of the experimental results.

Protein determination

The protein contents of the crude enzyme and the TPP fractions were determined using the Bradford method (Bradford, 1976) with BSA as a protein standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) on a Biorad Mini Protean electrophoresis unit. The gel was stained with Coomassie Brilliant Blue R-250 for 1 h and then de-stained with 40% methanol and 10% acetic acid for 2 h.

Determination of cellulase activity

Cellulase activity, in its free and immobilized forms, was measured using the method described earlier. One unit of enzyme was defined as the amount of enzyme required to produce 1 µmol min^{-1} of reducing sugar under assay conditions. The amount of reducing sugar produced was estimated using the DNS method (Miller, 1959). The CMC activity measurements were conducted five times and duplicated independently, and the mean values were used as the final activity values. The standard errors calculated for the mean values were <4%.

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Modification of bentonite and immobilization of cellulase

X-ray diffraction (XRD) analyses of the original, Na- and organo-bentonite were performed using a Bruker D8 Discover diffractometer with 2.2 kW Cu-*X* radiation. The samples were scanned in the range of 3–9°2θ, with a step size of 0.02° and a time per step of 0.5 s. Fourier-transform infrared (FTIR) spectroscopy analysis of the samples was performed using a Bruker Tensor 27 FTIR spectrometer in the range of 4000 to 400 cm^{-1}. Quartz and feldspar impurities were separated from the original bentonite by dispersion in water (10%, g mL^{-1}) with regular stirring at 1 h intervals overnight. Then, 1 M NaCl solution was added to the bentonite suspension. The suspension was stirred at 200 rpm overnight, followed by decantation. The decanted solution was centrifuged at 9000 rpm and the supernatant was discarded. The solid portion was washed until a negative test for chloride in the solution was obtained. After drying at 100°C, the Na-bentonite was mixed with 20% HDMAB suspension (5×10^{-3} M of HDMAB in ethanol-water) at room temperature for 2 h, followed by centrifugation at 9000 rpm and decantation of the supernatant (Ghiaci *et al.*, 2009a).

The organo-bentonite and the original bentonite (5 g) were separately dispersed in 5 mL of cellulase solution at 4°C for 1 h. The immobilized cellulase was separated from the free enzyme by centrifugation and washed with distilled water to remove any remaining unadsorbed soluble enzyme. Filtrates, as well as pellets, were collected for further experiments.

Determination of the optimum conditions of the immobilization parameters

The effects of the immobilization parameters on the activity of the cellulase were studied and the parameters were optimized to achieve high immobilization efficiency. The time of treatment with HMDAB (0.5–48 h), the amount of enzyme and the amount of bentonite used were varied in order to achieve the highest yield through immobilization. Cellulase activity measurements were conducted five times and were duplicated independently, and the mean values were used as the final activity values. The standard errors calculated for the mean values were <4%.

Reusability of the immobilized enzyme

The reusability of the immobilized cellulase was determined through standard assay conditions. The immobilized enzyme was recovered through decantation after each activity experiment, followed by washing five times with buffer to prepare the enzyme for the next batch. All experiments were performed in duplicate and the results represented the mean values of three independent experiments. The residual activity of the enzyme was calculated by considering the enzyme activity of the first cycles as being equal to 100%.

Operational stability of the immobilized enzyme

The operational stability of the immobilized enzyme was determined through CMC. The CMC was hydrolysed at 50°C using 50 mL of the substrate (i.e. 1% CMC in 50 mM Tris-HCl buffer (pH 7) and 0.1 g of immobilized cellulase). At the end of each reaction (80 min), homogenous samples were collected and subjected to centrifugation (5000 rpm, 15 min, room temperature), and the supernatant obtained was utilized for determining the cellulase activity.

Determination of the optimum pH, temperature and kinetic parameters

Effect of pH and temperature

The determination of the optimum pH for both the immobilized and free cellulase enzyme was conducted using CMC substrate solution prepared in buffers with pH values ranging from 3.0 to 12.0. To determine the optimum temperature of the free and immobilized cellulase, the enzyme activities were measured at temperatures ranging from 25 to 60°C with 5°C intervals. The relative enzyme activity was calculated as a percentage with respect to the activity of the enzyme at the optimum pH and temperature (100%). Cellulase activity measurements were conducted five times and were duplicated independently, and the mean values were used as the final activity values. The standard errors calculated for the mean values were <4%.

The Michaelis–Menten constant (K_m) and V_{max} values for both the free and immobilized cellulase were calculated at the optimum conditions of the respective enzymes under initial reaction rates using the Lineweaver–Burk plot.

Thermodynamic parameters

The temperature dependence of the rate constant (below the inactivation temperature) of an enzyme-catalysed reaction may be expressed by the Arrhenius equation:

$$k = A^{-E_a/RT} \tag{1}$$

where k is the rate constant, A is the pre-exponential factor, R is the gas constant and T is the absolute temperature in Kelvin. The E_a is calculated from a plot of lnk *vs* 1/T.

The activation free energy ($\Delta G^{\#}$) of cellulase is the most important factor in determining the rate constant because decreasing $\Delta G^{\#}$ is the most important factor in accelerating enzymatic reactions. The free energy of transition state formation ($\Delta G^{\#}_{E-T}$) describes the required free energy to form the transition state. The free energy of substrate binding ($\Delta G^{\#}_{ES}$) describes the free energy of enzyme–substrate destabilization. The thermodynamic parameters for the free and immobilized enzymes were calculated using the following equations derived from Eyring's transition state theory (Segel, 1975):

$$\Delta G^{\#} = -RT\ln(k_{cat}h/k_BT) \tag{2}$$

$$\Delta G^{\#}_{ES} = -RT\ln(1/K_m) \tag{3}$$

$$\Delta G^{\#}_{E-T} = -RT\ln(k_{cat}/K_m) \tag{4}$$

where R is the gas constant (8.314 J K^{-1} mol^{-1}), h is Planck's constant (6.63×10^{-34} J s) and k_B is the Boltzmann constant (1.38×10^{-23} J K^{-1}).

Results and discussion

Three-phase partitioning

The variable crude extract:t-butanol ratio effect on the partitioning of cellulase at a constant ammonium sulfate saturation (20%, m/v) is shown in Table 1. The greatest enzyme recovery (110%) and purification fold (3.6) were obtained at a 1:1 crude extract:

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t-butanol ratio in the aqueous phase. Usually, 0.2–0.5 mL of butanol mL^{-1} of starting crude sample is required for the precipitation of protein in the interfacial phase. In this study, the best result was obtained at a ratio of 1:1 (v/v) of crude enzyme:t-butanol. Although t-butanol is a branched alcohol and does not permeate into folded protein molecules and usually does not cause denaturation of the protein molecule (Roy & Gupta, 2002; Duman & Kaya, 2013b), when the ratio of sample:t-butanol exceeds 1, denaturation of the protein is more probable (Duman & Kaya, 2013b). Salt effects on the partitioning of cellulase were examined at a 1:1 (v/v) ratio of crude enzyme:t-butanol for the aqueous and interfacial phases. Generally, experiments start at a minimum salt concentration of 20% (m/v) to optimize the partitioning conditions (Dennison & Lovrein, 1997). In this study, salt experiments were also started with a minimum salt saturation. Recovery and purification fold increased in the interphase with increasing salt saturation up to 40% (m/v). Salt concentrations >50% (m/v) resulted in the reduction of recovery and purification fold, which may be due to irreversible denaturation of the protein (Duman & Kaya, 2013a). The TPP depends heavily on the saturation of ammonium sulfate (Table 2).

Finally, Table 3 summarizes the purification profile of *B. methylotrophicus* Y37 cellulase. In some cases, precipitation of proteins using TPP results in greater catalytic efficiency. Due to structural changes in the protein after TPP, the enzyme molecule has great flexibility. The treated protein with increased conformational flexibility shows greater catalytic activity compared to the untreated protein (Pike & Dennison, 1989; Duman & Kaya, 2013a). According to Duman *et al.* (2016), conventional purification methods for the cellulase enzyme resulted in low enzymatic yields. However, using the TPP method, the activity recovery of cellulase increased by 1.55-fold compared with the untreated enzyme. Figure 1 shows SDS-PAGE results for cellulase. The molecular weight of *B. methylotrophicus* Y37-derived cellulase was 50 kDa, which is in agreement with previous work (Duman *et al.*, 2016).

Table 1. Effect of various crude extract:t-butanol ratios on the recovery and purification fold in the interfacial phase and aqueous phase of cellulase from *B. methylotrophicus* Y37.

Crude extract:t-butanol ratio	Activity recovery (%)	Purification fold
1.0:0.5	85	2.5 ± 0.56
1.0:1.0	110	3.6 ± 0.71
1.0:1.5	70	1.1 ± 0.33
1.0:2.0	50	0.8 ± 0.45

Saturation amount of ammonium sulfate was fixed to 20% (w/v) and the crude extract: t-butanol ratio was varied in the range of 1.0:0.5–1.0:2.0. Both the aqueous phase and the interfacial precipitate were collected and analysed for protein content and activity.

Table 2. Effect of various ammonium sulfate saturations on the purification fold and recovery in the interfacial phase of cellulase from *B. methylotrophicus* Y37.

Ammonium sulfate saturation (m/v %)	Activity recovery (%)	Purification fold
20	110	3.6 ± 0.71
30	140	4.2 ± 0.23
40	155	5.8 ± 0.48
50	85	2.1 ± 0.59
60	45	0.9 ± 0.75
70	20	0.5 ± 0.36

Various amounts of ammonium sulfate (20%, 30%, 40%, 50%, 60%, 70% m/v) were added to the crude extract. The crude extract:t-butanol ratio was chosen as 1.0:1.0 (v/v) according to the results from Table 1; the aqueous and interfacial phases were collected and analysed for protein content and activity

Fourier-transform infrared spectroscopy results

Figure 2 shows the FTIR spectra of the original bentonite, Na-bentonite, organo-bentonite and immobilized cellulase. In Fig. 2a, the two bands at 3696 and 3620 cm^{-1} may be assigned to Si–OH and Al–Al–OH stretching (Dutta & Singh, 2015). Furthermore, the broad band at ~3383 cm^{-1} may be attributed to the –OH stretching vibration of inter-layer water in the smectite. The band at 1629 cm^{-1} corresponds to the –OH bending of smectite interlayer water (Khenifi *et al.*, 2007). In addition, the intense band at 1010 cm^{-1} is due to Si–O–Si stretching (Aytas *et al.*, 2009). The remaining bands at 916, 842 and 696 cm^{-1} are attributed to the Al–Al–OH, Al–Mg–OH and Al–O–Si vibrations of smectite, respectively, whereas the band at 792 cm^{-1} is indicative of free quartz impurity (Chen *et al.*, 2011; Kumararaja *et al.* 2017). The FTIR spectrum for Na-bentonite (Fig. 2b) is similar to the original sample except for the slight shift of the bands (Jović-Jovičić *et al.*, 2008). The FTIR spectrum of organo-bentonite (Fig. 2c) contains new bands at 2927 and 2854 cm^{-1} attributed to the symmetric and asymmetric stretching vibrations of the methylene and methyl group ($-CH_2$, $-CH_3$) of the aliphatic chain of HDMAB (Parolo *et al.*, 2014). In addition, the new band at 1543 cm^{-1} may be assigned to the bending vibrations of the alkyl chain (Jović-Jovičić *et al.*, 2008), indicating the modification of smectite by HDMAB. Finally, the spectrum of the organo-bentonite with immobilized cellulase enzyme does not show characteristic bands of the cellulase enzyme (Fig. 2d). This may be due to the overlapping of the organo-smectite and cellulase bands. The low weight ratio of cellulase to organo-bentonite may also be a reason for this behaviour. However, small changes in the spectrum are visible. The broad band at ~3380 cm^{-1}, attributed to the –OH stretching vibration of inter-lamellar water in smectite, shifted to 3350 cm^{-1} due to the –NH and –OH stretching vibrations of the amino and OH groups in cellulase. The mode at 1467 cm^{-1} may be assigned to the C = O stretching of the carboxyl group and the –OH bending vibration of the alcohol (Pei *et al.*, 2010; Zhao *et al.*, 2019). It may be concluded that the cellulase enzyme was successfully immobilized to organo-bentonite.

X-ray diffraction results

The XRD traces of the original bentonite, Na-bentonite and organo-bentonite dried in a drying oven and stored in desiccators are shown in Fig. 3. The original smectite displayed a d_{001} of 1.44 nm, which shifted to 1.23 nm after Na saturation. After modification of bentonite with monolayer coverage (5×10^{-3} M HDMAB in ethanol-water, bentonite–smectite monolayer (BMS)), the d_{001} increased to 1.74 nm, indicating that the surfactant ions were intercalated in the interlayer space (Favre & Lagaly, 1991; Homaei, 2015).

The interlayer distance of smectite increased in organo-bentonite, and as a result, the *B. methylotrophicus* Y37-derived cellulase enzyme was able to gain access between the smectite layers. Bentonite modified with cetyltrimethylammonium bromide (CTMAB) showed d_{001} values of 1.38 nm for Na-smectites (BMS-smectite monolayer) and 1.46 nm after immobilization of α-amylase enzyme by smectite (Homaei, 2015). In another study, Na-smectites (BMS-smectite monolayer) showed d_{001} values of 1.38 nm, which decreased to 1.32 nm after

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Table 3. TPP purification and recovery profile of cellulase from *B. methylotrophicus* Y37.

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification (fold)	Recovery (%)
Crude extract	90.32	98.18	0.92	1.0	100
Interfacial phase of TPP	140.00	26.12	5.36	5.8	155

Crude extract from *B. methylotrophicus* Y37. The interfacial phase of TPP consists of crude extract:t-butanol at 1.0:1.0 and 40% ammonium sulfate saturation at pH 7.

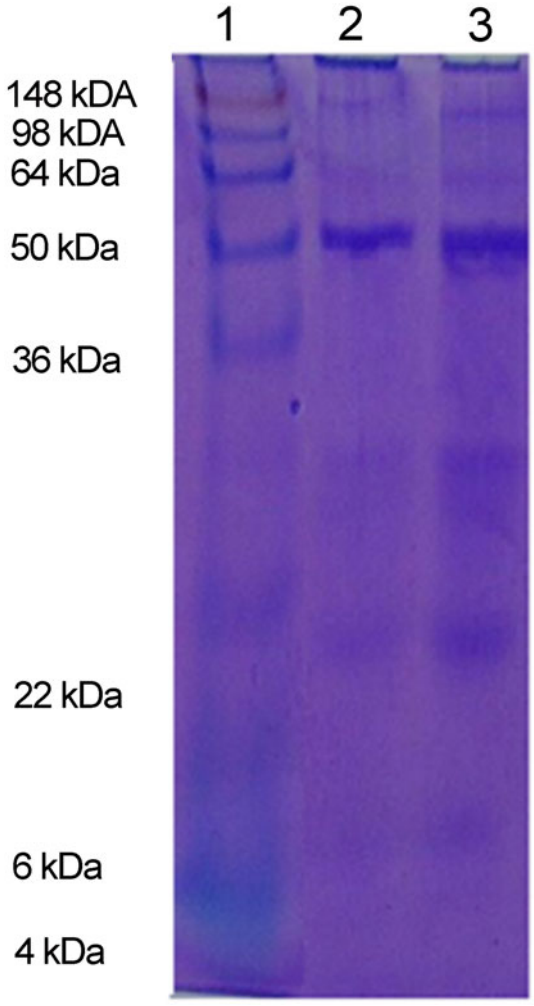

Fig. 1. SDS-PAGE analysis of recovered and purified cellulase from *B. methylotrophicus* Y37. Lane 1: marker protein (20 µg); lane 2: purified cellulase (20 µg); lane 3: crude extract of cellulase (20 µg).

immobilization of alkaline phosphatase (Ghiaci *et al.*, 2004). Moreover, the diffraction maximum of sepiolite modified with CTMAB decreased from 1.176 to 1.156 after immobilization of the enzyme alkaline phosphatase by sepiolite (Ghiaci *et al.*, 2009a). It follows that the molecular weight of the enzyme that was immobilized and the degree of extension of the interlayer spaces in clays are dependent on the structure of the support material used.

The chemical composition of bentonites was determined by X-ray fluorescence spectrometry using a Bruker S8 Tiger (Table 4). The CaO and MgO contents MgO of Na-bentonite decreased with respect to raw bentonite after Na exchange. In addition, the Na_2O content in organo-bentonite decreased significantly compared to Na-bentonite.

Optimization studies on the immobilized enzyme

Immobilized cellulase enzymes have several industrial applications, such as in pharmaceuticals, environmental industries and particularly in textiles. Enzymatic hydrolysis of lignocellulosic biomass by cellulase enzymes is considered to be an encouraging route in bioethanol production. However, the decrease in enzyme activity after its use is a critical limitation of this approach (Ghiaci *et al.*, 2009b; Ingle *et al.*, 2017). Few studies are available in the literature on the immobilization of *B. methylotrophicus* Y37-derived cellulase on organo-bentonite through non-covalent interactions. In the present study, therefore, the target enzyme was immobilized on modified organo-bentonite.

The reason for selecting bentonite for the immobilization, in addition to its low cost, was the fact that it is possible to vary the surface properties (hydrophilicity or hydrophobicity) of bentonite through ion exchange. The critical micelle concentration of HDMAB, which is a cationic surfactant, has been reported to be 1×10^{-3} mol dm^{-3}. Use of the cationic surfactant at concentrations lower than the critical micelle concentration results in the retention of surfactant monomers through ion exchange, forming a monolayer (BMS, smectite monolayer) (Ghiaci *et al.*, 2009a). Increasing the surfactant concentration to a value greater than the critical micelle concentration leads to interaction between the hydrocarbon tails of the surfactant, which results in the formation of a double layer (BBS, smectite bilayer) (Ghiaci *et al.*, 2009a). Therefore, in the present study, 5×10^{-4} M (BMS) and 5×10^{-3} M (BBS), which are the lower and upper concentrations of the critical micelle concentration respectively, were studied. However, as BBS concentrations did not permit reuse of the enzyme, only BMS concentrations, which formed a monolayer of HDMAB, were continued in subsequent experiments.

In the first step of the present optimization study, Na-bentonite was treated with 5×10^{-4} M HDMAB at room temperature for 0.5, 1, 2, 3, 24 and 48 h. The greatest immobilization yield was observed after 2 h of treatment (Fig. 4). The 100% value depicted in Fig. 4 represents the activity of the free enzyme. The immobilization yield obtained at 2 h of HDMAB treatment was 65%.

In a previous study of the immobilization of the invertase enzyme on bentonite through non-covalent interactions, the treatment time of bentonite with HDMAB was extended to 24 h at room temperature (Andjelkovic *et al.*, 2015). In a similar study on the immobilization of the lipase enzyme by bentonite, CTMAB as the surfactant was used to treat bentonite for 15 h at 60°C (Dong *et al.*, 2013). In comparison with the aforementioned studies, the duration for bentonite modification used in the present study was moderate, indicating that cation exchange occurs within a shorter period of time. This might be considered an advantage for commercial applications.

Figure 5 shows the immobilization efficiency of 5 g of modified bentonite with various concentrations of the cellulase enzyme. The greatest, of 94%, was obtained with a 5.17 mM (5 mL) concentration of the enzyme. As organo-bentonite does not get sufficiently wet at volumes below 4 mL (4.13 mM), no further attempts were made to decrease the amount of enzyme in the experiment.

As the amount of enzyme increased, the amount of enzyme immobilized on the support material also increased. The saturated

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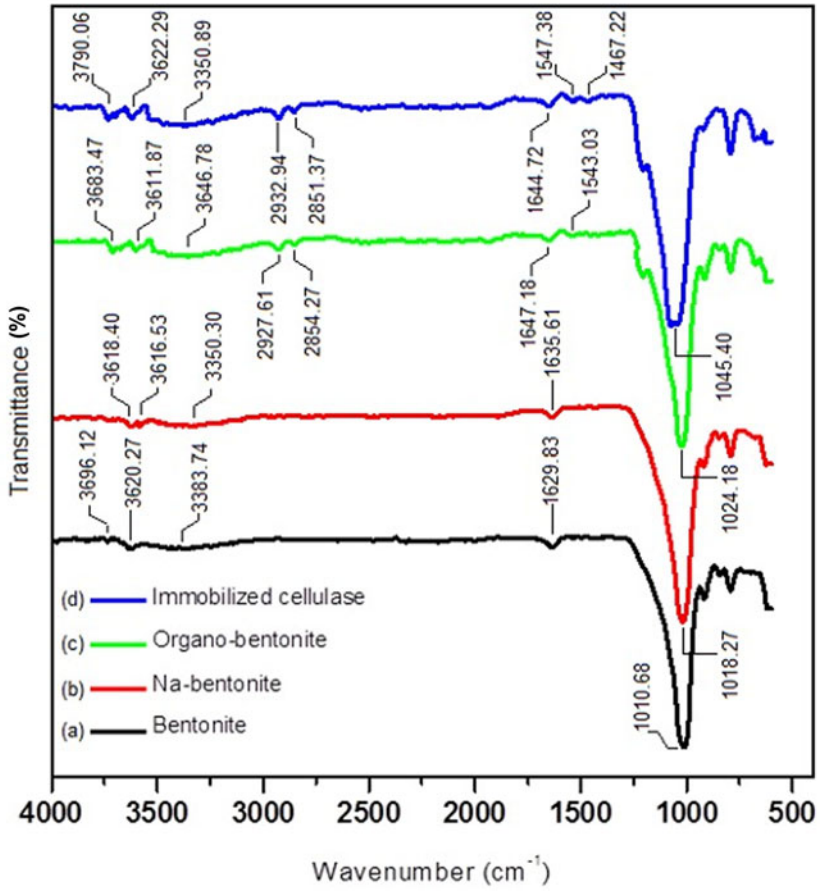

Fig. 2. FTIR spectra of (a) raw (pristine) bentonite, (b) Na-bentonite, (c) organo-bentonite and (d) immobilized cellulase.

cellulase concentration determined in the present study was 140 units. The decreasing immobilization yield with increasing enzyme concentrations might be due to the aggregation of the enzyme molecules on the surface of smectite particles. This might occur due to the substrate having potentially increased the energy barrier for accessing the active region of the enzyme molecule (Kumar *et al.*, 2009).

Various amounts (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 g) of modified bentonite were treated with 5.17 mM of cellulase enzyme at 4°C for 2 h. Figure 6 depicts the graph of optimization against the amount of bentonite used. The 100% value depicted in Fig. 6 represents the activity of the free enzyme. While a yield of 18% was obtained with an initial amount of 0.5 g of modified bentonite, the immobilization efficiency increased gradually with increasing amounts of organo-bentonite, reaching 94% with 5 g of modified bentonite (Fig. 6).

Biotransformation of CMC by the immobilized cellulase enzyme

The CMC and the total CMC content of the bulk reaction medium were obtained by calculating the immobilized cellulase enzyme biotransformation (Fig. 7). Biotransformation of the 16.1 µM concentration of CMC was completed in 80 min.

Reuse of the immobilized enzyme

One of the most important advantages of the immobilization process is enzyme recycling by providing the enzyme with a solid support for attachment. The reuse of the cellulase enzyme immobilized with modified organo-bentonite is depicted in Fig. 8. The 100% value in Fig. 8 represents the activity of the free enzyme. The enzyme retained its activity for 20 activity cycles. The activity observed in the 25th experiment was 68%. A few researchers have reported previously the use of carbodiimide-activated Fe_3O_4 nanoparticles for the covalent immobilization of cellulase. The immobilized enzyme was observed to retain 30.2% of the initial activity and was successfully reused six times with a small decrease in activity after each use (Liaoa *et al.*, 2010). In a similar study, cellulase was immobilized on polyvinyl alcohol/Fe_2O_3 magnetic nanoparticles, whereby the immobilized cellulase maintained 40% of its initial activity after four cycles (Tu *et al.*, 2006) and 35% of its initial activity after 15 cycles (Zhang *et al.*, 2016).

Characterization of the free and immobilized enzyme

Determination of optimum pH and temperature for the free and immobilized enzymes

In order to analyse the effect of pH on the free and immobilized enzymes, activity analyses were performed using substrates prepared in 50 mM buffer solution in the pH range 3–12. The relative activities according to the pH values were calculated with respect to the highest activity observed (100%), and the results were plotted as the maximum activity *vs* pH (Fig. 9).

The optimum pH for the free enzyme was 5 and that for the immobilized enzyme was 6. In general, enzymatic activity is known to be affected substantially by pH changes in the reaction

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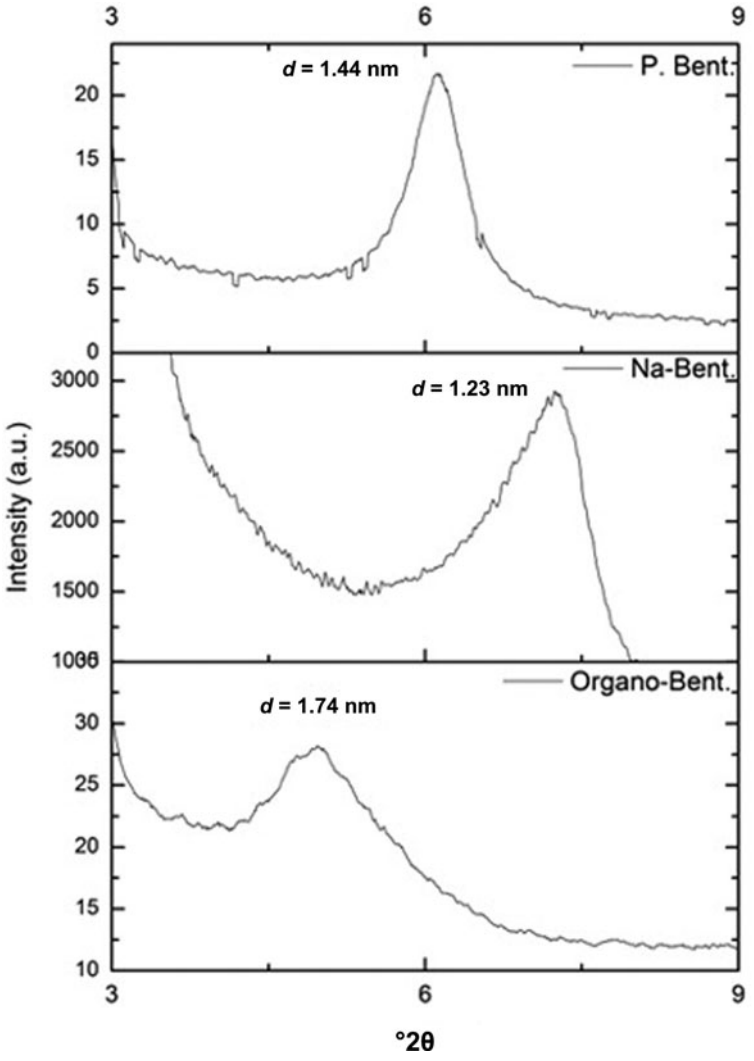

Fig. 3. XRD traces of raw (pristine) bentonite (P. Bent.), Na-bentonite (Na-Bent.) and organo-bentonite (Organo-bent.).

Table 4. Chemical composition of raw, Na- and organo-bentonites.

	Raw bentonite	Na-bentonite	Organo-bentonite
LOI	6.50	1.62	10.26
SiO_2	73.00	73.00	72.00
Al_2O_3	13.00	11.3	12.80
Fe_2O_3	0.70	0.73	0.87
TiO_2	0.05	–	0.07
CaO	1.10	0.33	0.50
MgO	3.00	2.08	2.30
Na_2O	0.25	6.15	0.58
K_2O	1.00	0.62	0.56
Cl	–	4.17	0.06

LOI = loss on ignition.

medium due to the flexibility of enzymes. While enzyme immobilization may assist in enhancing pH stability, the optimal pH for the enzyme may change after immobilization due to a change in the microenvironment of the enzyme. Therefore, activities of the enzyme at various pH values prior to and after immobilization should be compared (Sedaghat *et al.*, 2009). The differences in the optimum pH values in the free and immobilized enzymes depend on the interactions between the enzyme and the support material. Electrostatic interactions between the charges on the surface of the support material and those on the surface of the enzyme modify the charge at the microenvironment of the enzyme, which leads to pH shifts in the enzyme molecule (Gao *et al.*, 2018). As covalent immobilization stabilizes the enzyme to the solid support, immobilized cellulase has been reported to exhibit high relative activity over a wide pH range compared to the free enzyme. Indeed, Tao *et al.* (2016) reported that immobilized cellulase showed relatively high activity in the same pH range compared to free cellulase, although the optimal pH did not change. This increase was associated with the microenvironment of the immobilized enzyme. In the present study, the optimum pH value observed for the free enzyme was 5 and that for the immobilized enzyme was 6. The electrostatic interactions that might have occurred in the *B. methylotrophicus* Y37-derived cellulase immobilized with organo-bentonite might have caused changes in the microenvironment of the enzyme, modifying the optimum pH of the enzyme.

The temperature of the immobilized enzyme is necessary for determining how immobilization affects the enzymatic activity (Dong *et al.*, 2012). In order to determine the effect of temperature on the free and immobilized enzymes, catalysis of CMC prepared in Tris-HCl buffer (pH 7.0) was investigated at various temperatures (25, 27, 30, 32, 35, 40, 45, 50, 55 and 60°C). The relative activities (%) of the enzyme at these temperatures were calculated with respect to the greatest activity obtained (100%)

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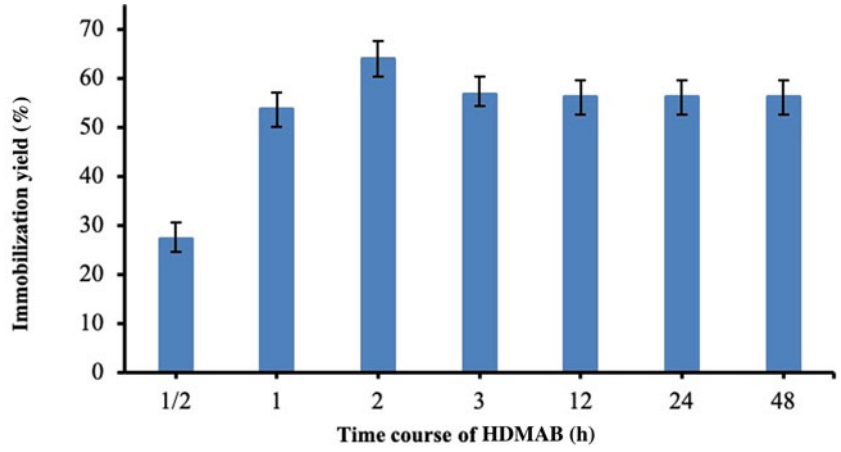

Fig. 4. The effect of HDMAB time treatment on the immobilization efficiency of 4 mL of cellulase with 5 g of organo-bentonite.

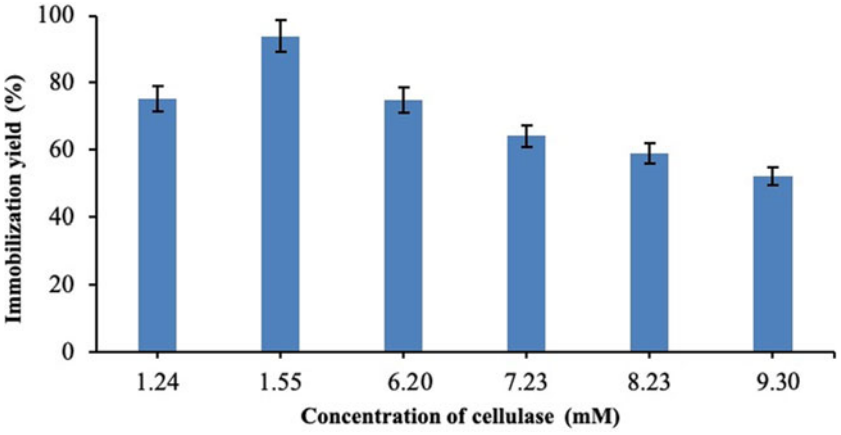

Fig. 5. Effect of increasing enzyme concentrations on immobilization efficiency of 5 g of organo-bentonite (activity: 112, 140, 168, 196, 224 and 252 U, respectively).

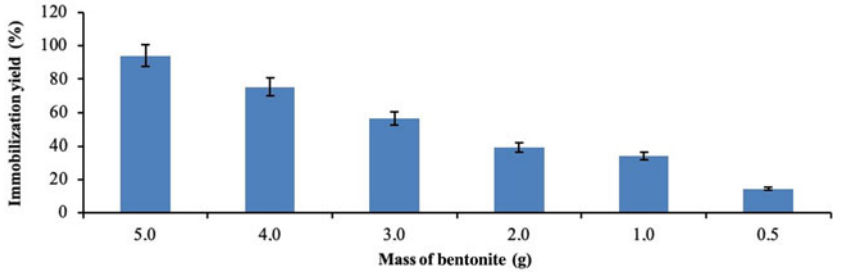

Fig. 6. Effect of increasing amounts of bentonite with 1.55 mM of enzyme on immobilization efficiency (specific activity: 153.02 U mg^{-1}).

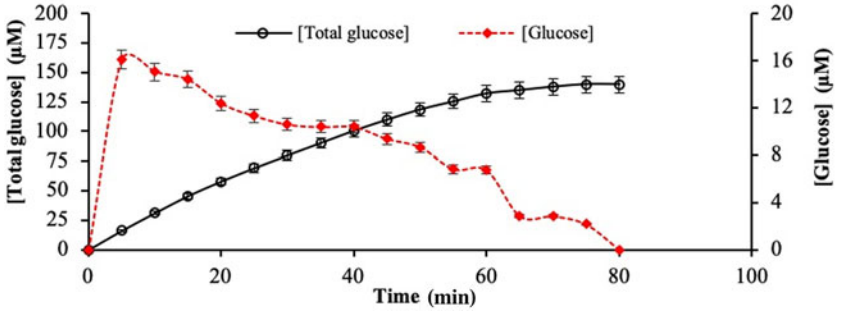

Fig. 7. Biotransformation of CMC by immobilized cellulase enzyme.

(Fig. 10). The optimum temperatures observed for the free enzyme and the immobilized enzyme were 45°C and 50°C, respectively.

Previous studies have investigated the effect of temperature on free and immobilized enzymes. Zhang *et al.* (2016) reported that the optimum temperature for the free enzyme and the

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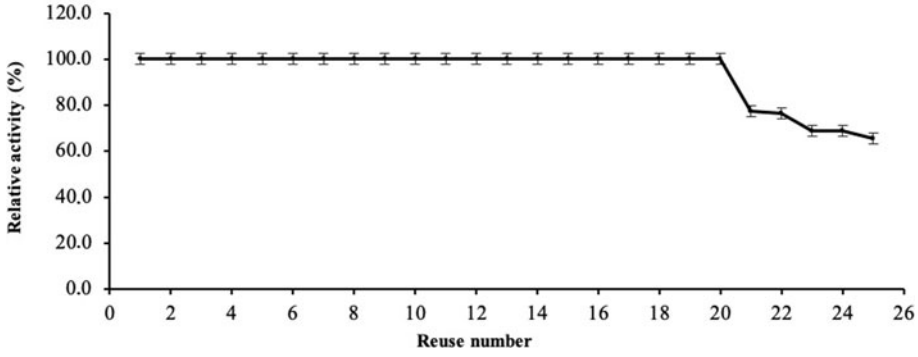

Fig. 8. Reuse of immobilized cellulase.

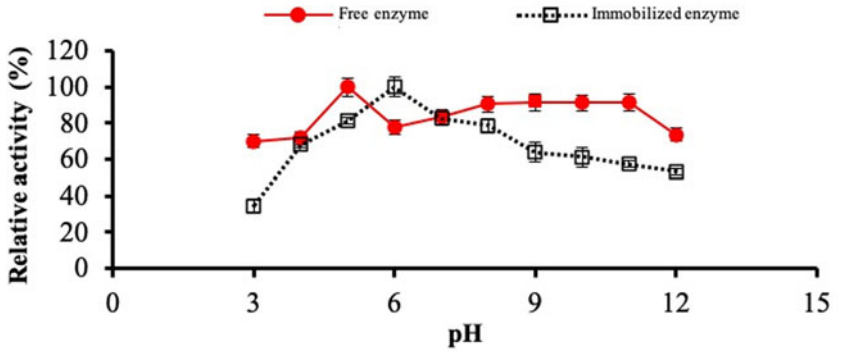

Fig. 9. Effect of pH on the free and immobilized enzymes.

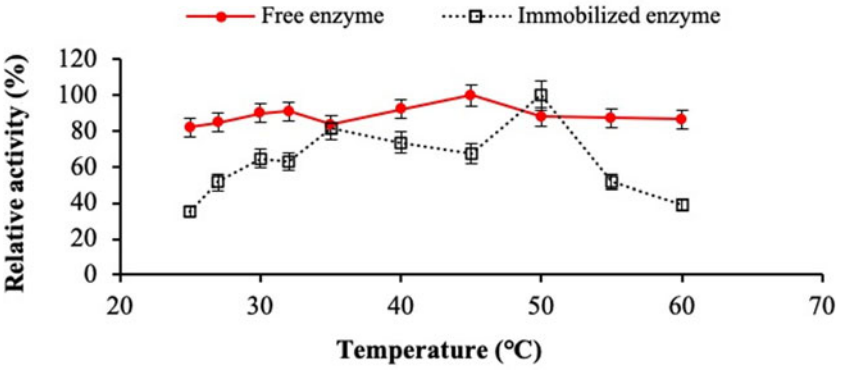

Fig. 10. Temperature effect on the free and immobilized alkaline proteases.

immobilized enzyme during the immobilization of acidic cellulase enzyme isolated from *Synergistes* spp. with activated carbon particles was 50°C. The optimum temperature for the lipase enzyme on bentonite through non-covalent interactions was 30°C for both the free and immobilized enzymes (Dong *et al.*, 2012, 2013). Similarly, the optimum temperatures for the pectinesterase enzyme on bentonite were 60°C and 50°C for the free enzyme and the immobilized enzyme, respectively (Karakuş *et al.*, 2008). In the present study, the optimum temperature for the *B. methylotrophicus* Y37-derived cellulase as a free enzyme was 45°C and that for the immobilized form of the enzyme was 50°C. This small change observed in the optimum temperatures of the free and the immobilized enzymes might be due to a change in the electrostatic interactions in the conformation of the enzyme upon the immobilization of the enzyme with organo-bentonite. Although the changes in the optimal temperature indicate conformational changes in the enzyme, the present study demonstrated that the stability of the enzyme increased by 5°C with immobilization, indicating improved thermal stability of the enzyme.

Determination of the activation energies for the free and immobilized enzymes

The E_a values for the free and immobilized enzymes were determined from the experimental rate constants or diffusion coefficients at various temperatures (Fig. 11). The E_a values for the free and the immobilized enzymes were 3.49 and 1.80 kJ mol^{-1}, respectively (Table 5). Although it has been reported that multipoint interactions of the cellulase molecule with the support might cause a decrease in the conformational flexibility of the enzyme, thereby resulting in greater E_a values (Mateo *et al.*, 2007; Kumari *et al.*, 2015), there are also studies that report an increase in the conformational flexibility of the enzyme (Ahmad & Khare, 2018).

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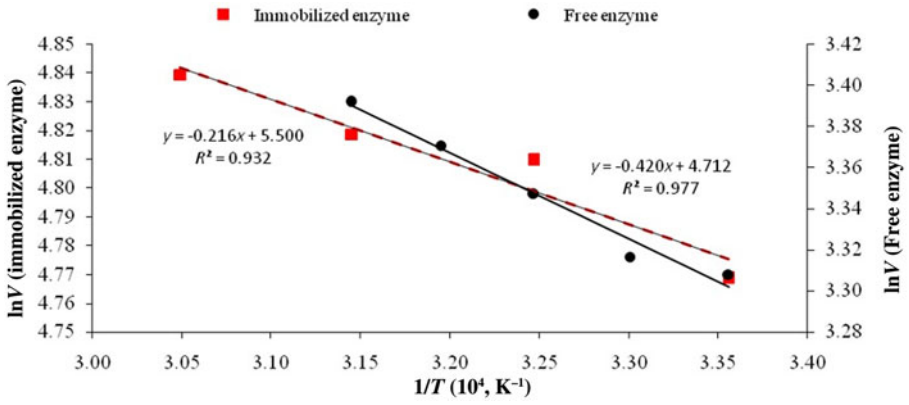

Fig. 11. Activation energies of the free and immobilized enzymes.

Table 5. Kinetic parameters for the free and immobilized cellulase.

Parameter	Free enzyme	Immobilized enzyme
K_m (mg mL^{-1} CMC)	0.19 ± 0.025	0.375 ± 0.048
V_m (U mL^{-1} min^{-1})	7.42 ± 0.52	156.25 ± 2.51
k_{cat} (min^{-1})	9275 ± 98.14	19531.5 ± 146.42
k_{cat}/K_m (mL min^{-1} mg^{-1})	4.78 × 10^4 ± 143.56	5.21 × 10^4 ± 153.78
E_a (kJ mol^{-1})	3.49 ± 0.55	1.80 ± 0.15

Determination of the K_m and V_m values for the free and immobilized enzymes

The Lineweaver–Burk plot utilized to calculate the K_m and V_m values for the free and immobilized enzymes is depicted in Fig. 12, and the K_m and V_m values are listed in Table 5. The microenvironment of the immobilized enzyme molecules is important for determining the behaviour of the enzyme (Li *et al.*, 2012). In the present study, the K_m and V_m values of the organo-bentonite-immobilized cellulase increased by 1.93- and 21-fold, respectively, compared to the values of the free enzyme. The increase in the K_m value of the immobilized enzyme could be related to the decrease in the affinity of the enzyme towards its substrate, which might have occurred because of changes in the electrostatic interactions and possible conformational changes in the microenvironment of the enzyme due to immobilization (Li *et al.*, 2012; Gao *et al.*, 2018). Increases in the V_m value of an enzyme post-immobilization are not common in the literature, although some evidence of this does exist (Roy *et al.*, 2003; Sedaghat *et al.*, 2009; Li *et al.*, 2012; Zhang *et al.*, 2016). Increases of the V_m value of the immobilized cellulase in comparison to that of the free cellulase might be attributed to the increased stability of the enzyme post-immobilization. When the enzyme is saturated with a substrate, the k_{cat} value is defined as the amount of substrate that is converted to the product in unit time, with the k_{cat}/K_m value representing the catalytic performance of the enzyme. The k_{cat} and k_{cat}/K_m values of the organo-bentonite-immobilized cellulase increased by 21- and 11-fold, respectively, compared to the values of the free enzyme. Saleem *et al.* (2005) conducted a study on the immobilization of *Arachniotus citrinus*-derived endoglucanase with polyacrylamide gel, and they reported k_{cat} values for CMC hydrolysis of 80 and 240 min^{-1} for the free and the immobilized enzymes, respectively. These authors reported that the electrostatic changes that occurred as a result of the immobilization increased the affinity of the enzyme towards CMC, the substrate of the enzyme. As a result of these electrostatic changes, the reaction rate of the enzyme increased as the substrate molecule of CMC was more strongly bound to the active site of the enzyme (Saleem *et al.*, 2005). This finding is related to the fact that the binding energy of the enzyme–substrate complex is greater than the energy of the enzyme transition state, which leads to decreased k_{cat} and K_m values. In a previous study conducted on the immobilization of sulfide oxidase with diethylaminoethyl cellulose, the k_{cat} value increased by two-fold as a result of the immobilization of the enzyme, and this immobilization increased the affinity of the enzyme towards its substrate (Mohapatra *et al.*, 2008). In the present study, immobilization of *B. methylotrophicus* Y37-derived cellulase with organo-bentonite resulted in a 21-fold increase in the k_{cat} value for the immobilized enzyme in comparison to the free enzyme. As a result, it is argued that the electrostatic changes resulting from the immobilization process increase the interactions between the enzyme and its substrate, thereby increasing the catalysis rate. Li *et al.* (2012) observed that the k_{cat}:K_m ratio of the immobilized neutral protease enzyme was greater than that of the free neutral protease enzyme. These authors reported that the immobilization support that offered the best k_{cat}:K_m ratio would be the most suitable choice for the immobilization process. Similarly to the findings in the literature, in the present study, the k_{cat}:K_m ratio of the immobilized *B. methylotrophicus* Y37-derived cellulase enzyme was 11-fold greater than that of the free enzyme. This finding confirmed the increased post-immobilization catalytic performance of the enzyme used.

Determination of the thermodynamic parameters of the free and immobilized enzymes

The $\Delta G^{\#}$ and $\Delta G^{\#}_{E-T}$ determined for the immobilized enzyme were decreased in comparison to those obtained for the free enzyme. On the other hand, the value of $\Delta G^{\#}_{ES}$ was increased (Table 6).

The changes in $\Delta G^{\#}$ for substrate hydrolysis indicated the possibility of an enzymatic reaction, such as the conversion of a transition state complex ($ES^{\#}$) into the product. The lower $\Delta G^{\#}$ value obtained for the immobilized cellulase enzyme implied that the conversion of its transition complex into products was more spontaneous in comparison to that of the free enzyme. The $\Delta G^{\#}_{ES}$ reaches its greatest value when all of the binding groups on the substrate molecule match the binding site of the enzyme (enzyme–substrate complementarity). However, the complementarity between the enzyme and the transition state form of the substrate ($S^{\#}$) is more beneficial to the spontaneity of the biochemical reaction. In such cases, due to the conformational change in the substrate molecule, the $\Delta G^{\#}_{ES}$ increases, whereas the $\Delta G^{\#}$ of the

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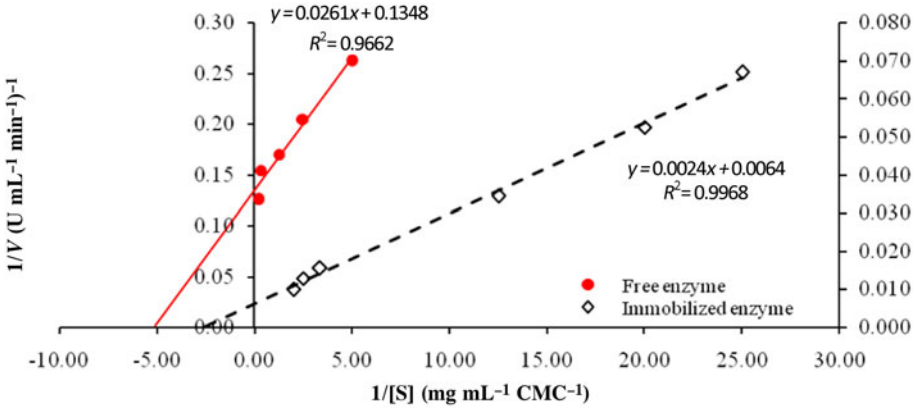

Fig. 12. Lineweaver–Burk plot of the free and immobilized enzymes.

Table 6. Thermodynamic parameters for the free and immobilized cellulase.

Parameter	Free enzyme	Immobilized enzyme
$\Delta G^{\#}$ (kJ mol^{-1})	65.78 ± 2.95	57.60 ± 4.56
$\Delta H^{\#}$ (kJ mol^{-1})	0.81 ± 0.078	−0.89 ± 0.56
$\Delta S^{\#}$ (kJ mol^{-1} K)	−0.20 ± 0.015	−0.18 ± 0.035
$\Delta G^{\#}_{E-T}$ (kJ mol^{-1})	28.94 ± 4.72	−35.35 ± 3.32
ΔG_{ES} (kJ mol^{-1})	−4.40 ± 0.96	−2.63 ± 0.53

reaction decreases. If the substrate in its original conformation is complementary to the enzyme molecule, the $\Delta G^{\#}_{E-T}$ decreases, while the $\Delta G^{\#}$ increases (Duman *et al.*, 2014). The latter case is compatible with the results of the present study, where the $\Delta G^{\#}_{ES}$ value of the enzyme increased, while the $\Delta G^{\#}_{E-T}$ and $\Delta G^{\#}$ values of the immobilized cellulase enzyme decreased, indicating an increase in the spontaneity of the biochemical reaction post-immobilization. Finally, the ΔH is related to the energy necessary for the formation of the transition state. The lower ΔH value obtained for the immobilized enzyme in the present study in comparison to that of the free enzyme implied a lower energy for the formation of the transition state, and thus convenient product formation. The ordered structure of an enzyme is identified through entropy. In general, the lower entropy (ΔS) obtained for the immobilized cellulase enzyme indicated that the immobilized form of the enzyme was more ordered than its free form.

Conclusion

Bacillus methylotrophicus Y37-derived cellulase was purified using TPP. The recovery activity of enzyme was greater than that of the purified enzyme, which was purified using conventional purification methods. The immobilization of *B. methylotrophicus* Y37-derived cellulase enzyme with bentonite was also investigated. As a result of this immobilization, both the E_a and the catalytic performance increased. One of the most important parameters in enzyme immobilization studies is the reusability of the enzyme. In the present study, the activity of the enzyme was maintained after 25 activity cycles. In the 25th experiment, 68% of the initial activity was retained. The yield obtained after a 2 h of immobilization of 5 mL of enzyme with 5 g of organo-bentonite was 90%.

Bentonite is a suitable support material with potential for industrial applications as it provides high yields, enables reusability of the enzyme and has high catalytic performance. Bentonite may also be preferred because of its abundant availability, accessibility and low cost. According to the findings of the present study, bentonite is suitable for use as a support material for enzyme immobilization at an industrial scale.

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