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Immobilization of Cellulases Produced by *Penicillium brevicompactum* AUMC 10987, using Cross-Linkage, Chitosan-Coating and Encapsulation

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ABSTRACT

This study was designed to optimize different conditions for improving enzyme production by *Penicillium brevicompactum* AUMC 10987, and to precipitate and immobilize cellulases by cross-linking method using glutaraldehyde, magnetic nanocarriers and encapsulation using Ca-alginate. Maximum activity of cellulase was achieved in medium containing date palm leaves, sodium nitrate, after 9 days incubation at 30°C and pH 6. Solid state fermentation was more convenient method in cellulase production. After ammonium sulphate precipitation, enzyme activities exceeded two-folded, constructing more stability for the enzyme structure and giving maximum activities at 50℃ and pH 4.8. Hence, precipitated enzyme could work in higher temperature and acidity conditions. The immobilization of cellulases by encapsulation was the most valuable technique retaining the activity of enzyme. Moreover, the immobilized enzyme remained active up to 80°C, giving its maximum activity at 50°C and pH 5.5. Enzyme encapsulated within alginate beads is preferred due to easy for formulation, mild gelation conditions, non-toxic, biocompatibility, low cost and resistance to microbial attack. Magnetic nannocariers immobilized enzyme had opportunity to be reused after 4 cycles/16 hours. The current study could provide a robust and highly valuable immobilized enzyme for different industrial and biotechnological applications.

Keywords: Ca-alginate, Cellulase, Encapsulation, *Penicillium brevicompactum* AUMC 10987

INTRODUCTION

Cellulose is a water insoluble long linear polymer composed of repeated units of β-D-glucose (8000-12000 glucose units) interlinked by β-1,4 glycosidic bonds (Somerville, 2006). Because of its highly ordered structure, cellulose is very hard to be degraded and that may explain its unusable and accumulation in nature as a waste material. The capacity to degrade the natural cellulose implies the synthesis of the entire cellulolytic system (Devi and Kumar, 2012). Cellulase enzyme complex consists of three major enzyme components: (a) endoglucanases (CMCase) (endo-1,4-D-glucano-hydrolase, EC 3.2.1.4), hydrolyzing at random the internal glycosidic linkages of amorphous cellulose chain, (b) exoglucanases (FPase) (1,4-β-D-cellobio-hydrolase, EC 3.2.1.91), acting in a progressive manner on the reducing or non-reducing ends of cellulose chains and (c) β-glucosidases (EC 3.2.1.21), these collectively hydrolyze polymer chain of cellulose producing glucose units as simple sugar molecules. Cellulase enzymes play a major role in industrial applications (Kirk *et al.*, 2002). Also, microbial cellulase is used for bioremediation, waste water treatment and single cell protein (Alam, 2005). Cellulases have potential applications in pharmaceutical industry and biofuels production by fermentation of cellulosic biomass (Ali and Saad El-Dein, 2008). It was also proved that application of cellulolytic fungi improves the composting potential of cellulose waste, in addition (Hart *et al.*, 2002).

The current demands of sustainable green methodologies have increased the use of enzymatic technology in industrial processes. But, the harsh conditions of industrial processes, increase propensity of enzyme destabilization, shortening their industrial lifespan, as well as, the free enzymes usually have poor stability towards pH, heat or other factors and there is no possibility to recover and reuse (Kim *et al.*, 2006). There are also limitations in using enzymes in large-scale production such as high cost and lack of long-term operational stability. It is difficult to separate them from the reaction system, which limits the recovery of the enzyme and may lead to contamination of the final product (Vallet-Regi *et al.*, 2001). Therefore, there is a great demand for finding ways to improve enzyme stability and reusability. All these requirements may be guaranteed by enzyme immobilization. Immobilization technique, as a very powerful tool, has been intensively conducted to prepare various high performance and economically-feasible biocatalysts with improved stability and reusability for biotechnological applications. The immobilized enzymes can work in a broader pH and temperature range showing higher thermal stability than the free enzymes (Xu *et al.*, 2014).

The present work was designed to optimize the conditions to maximize cellulase production by *Penicillium brevicompactum* AUMC 10987; to immobilize cellulases using cross-linkage, chitosan coating and encapsulation to obtain robust enzyme with

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high quality for industrial and biotechnological applications was also investigated.

MATERIALS AND METHODS

Optimization of cellulase production

Fungal isolate tested

Penicillium brevicompactum AUMC 10987 was isolated from rotted apple fruit, collected from Sohag city, Egypt. The isolate was identified based on macroscopic and microscopic features following the key and description of Pitt (1979). Inoculum was prepared from the fungus grown on Czapek's agar medium (g/l; glucose, 10; $NaNO_3$, 3; KH_2PO_4, 1; KCl, 0.5, $MgSO_4.7H_2O$, 0.5, $FeSO_4$, 0.01; and agar, 15) at 28°C, for 7 days.

Optimization parameters for maximum enzyme activity

Carbon sources

Date palm leaves, wheat straw, corn cob, saw dust, filter paper and cellulose powder (10 g /25 ml medium) were separately inoculated with 4 ml spore suspensions (10^7 spores/ ml) and incubated at 28°C for 7 day**s** (Abo-State *et al.*, 2010).

Nitrogen sources

Each of the nitrogen sources: Ammonium nitrate, peptone, beef extract and yeast extract was added to Czapek's medium with replacing glucose by date palm leaves (as the best carbon source) (Gautam *et al.*, 2011). Fifty ml of the medium were dispensed into Erlenmeyer flask (250 ml) and inoculated with 1 cm diam agar mycelial disc of 7 days- old fungal culture. The flasks were incubated at 28°C for 7 days.

Temperature range

The fungus was grown on medium containing date palm leaves and sodium nitrate (as the best nitrogen source), and incubated at different temperatures (25, 30, 35, 40, 45 and 50°C) for 7 days.

Incubation period

The previous medium (containing date palm and sodium nitrate) was inoculated with *P. brevicompactum* AUMC 10987, incubated at the optimum temperature (30°C) and enzyme activity was assessed after 3, 6, 9, 12 and 15 days of incubation.

pH values

To determine the best pH value for enzyme activity, Different pHs of 3, 5, 6, 7 and 12 were tested after 9 days of incubation.

Enzyme assay

Cellulase activity was assayed by mixing 0.9 ml of 1% CMC (w/v) (pH 5.3) with 0.1 ml of the clear supernatant, the mixture was incubated at 50°C for 30 min (Bailey *et al.*, 1992). The reaction was stopped by adding 3 ml of 3, 5-dinitrosalicylic acid (DNS) and boiled for 10 min. After cooling, the developing colour was read at 540 nm using spectrophotometer model (UNICAM, Model Helios- Gamma). The liberated reducing sugar was quantified using glucose as a standard. One unit of cellulase is defined as the amount of enzyme that liberates 1 µmole of glucose equivalent per minute under the assay conditions (Ghose, 1987).

Submerged fermentation

Czapek's broth medium was used in which glucose was replaced by date palm leaves (the best carbon source). After sterilization, 250 ml Erlenmeyer flask (containing 150 ml medium) was inoculated with three agar discs of 7 days-old mycelial growth of *P. brevicompactum*, then, the flasks were incubated under shaking conditions (120 rpm) at 30°C for 9 days. Cultures were filtered and centrifuged at 5000 rpm for 10 min. The clear supernatants were kept for crude enzyme precipitation.

Solid state fermentation

One hundred grams of small segments (1 cm^2) of date palm leaves were mixed well with 70 ml distilled water and put in Erlenmeyer flask (one liter each). After sterilization, the flask was inoculated with five agar mycelial discs of *P. brevicompactum* strain and incubated at 30°C under static conditions for 20 days. Then, cultures were filtered in the presence of 50 mM sodium citrate buffer (pH 5.3) and centrifuged at 5000 rpm for 10 min and the clear supernatants were used for further studies (Abo-State *et al*., 2010).

Enzyme precipitation

Using organic solvents

Eight hundred ml of cold acetone or n-propanol were added separately drop wise to 200 ml clear supernatant from submerged fermentation using separating funnel with slow stirring for 48 hours under cooling, then kept for 2 hours at 4°C for complete enzyme precipitation and centrifuged for 10 minutes at 5000 rpm (Dalal *et al*., 2007). The supernatant was discarded and acetone or n-propanol was evaporated at room tempruture and the precipitate was collected, weighed and dissolved in 5 ml of 50 mM sodium citrate buffer (pH 4.8). The enzyme activity and protein content were estimated in the solution. The remaining precipitate was kept for enzyme immobilization.

Using ammonium sulphate

Ammonium sulphate (70%) was slowly added to 200 ml crude enzyme while gently stirring for 24 hours under cooling and kept overnight at 4°C for complete precipitation, then centrifuged at 5000 rpm for 15 minutes and the pellet was dissolved in citrate buffer for dialysis (Kalyani *et al*., 2015).

Dialysis

After precipitation, dialysis required to exclude high salted buffer crude enzyme, by placing the precipitate in dialysis bag and dialyzed against reverse osmotic water overnight at 4°C (Periyasamy *et al.*, 2016). The precipitated enzyme inside the bag was lyophilized for removing more salts and water.

Determination of protein content

Protein content of the precipitated enzyme was determined according to Lowry *et al.* (1951). The protein concentration was calculated using bovine serum albumin as standard.

Determination of precipitated enzyme activity

Filter paperase activity (FPase) assay

It was assessed by adding 0.1 ml precipitated enzyme solution (in 10% sodium citrate buffer) to 0.5 ml of 0.05 M Na-citrate (pH 4.8); 50 mg filter paper (Whatman No.1) strip (1.0 x 6.0 cm) and 0.4 ml distilled H_2O. The mixture was incubated at 50°C for 30 min, then the reaction was stopped by adding 3 ml DNS, boiled for 10 min and after cooling, the developing color was detected at 540 nm (Mandels *et al.*, 1976). The liberated reducing sugar was quantified using glucose as a standard. One unit of FPase is defined as the amount of enzyme that liberates 1 µmole of glucose equivalent per minute under the assay conditions (Ghose, 1987).

Carboxymethyl cellulase (CMCase*)*

The enzyme was assayed by mixing 0.9 ml of 1% carboxymethyl cellulose solution (CMC) prepared in 50 mM Na-citrate buffer (pH 5.3) (w/v) with 0.1 ml precipitated enzyme solution, the mixture was incubated at 50°C for 30 min (Bailey *et al.*, 1992). The enzyme activity was assessed as mentioned above.

ß-glucosidase assay

To evaluate ß-glucosidase, 0.9 ml of cellobiose mixed with 0.1 ml precipitated enzyme solution and the mixture was incubated at 50°C for 30 min and the enzyme activity was determined as the above (Ghose, 1987; Bailey *et al.*, 1992)**.**

Effect of pH and temperature on the activity of precipitated enzyme

The cellulase enzymes precipitated by ammonium sulphate were the most active one. Therefore, the effect of different temperatures (30, 40, 50, 60, 70 and 80°C) and pH values (3.5, 4, 4.5, 4.8, 5, 5.3, 5.5, 6, 6.5 and 7) was studied to determine the optimum conditions for maximizing the enzyme activities. The activities of precipitated cellulases (FPase, CMCase and β-glucosidase) were assessed.

Immobilization of crude enzyme

Cross-linking method using glutaraldehyde

A mixture of 10 ml enzyme solution and 10 ml chitosan solution (2% in acetic acid 2%) (pH 5.0) was added drop wise into 10 ml mixture of $NaHCO_3$ (10% w/v) and glutaraldehyde (5% w/v) (Yu *et al.*, 2006). The reaction mixture was gently stirred at room temperature then the immobilized enzymes were filtered and washed several times with 50 mM Na-citrate buffer solution. Enzymes activity and protein content were measured.

Magnetic Nanocarriers

Chitosan-magnetite nanocarriers were prepared by mixing 100 mg of magnetite powder (Iron oxide, Fe_3O_4) (15.2 ± 2.6 nm) in 20 ml of 1% chitosan solution (prepared in 2% acetic acid) (Krajewska, 2004). Then, 13.2 ml of 0.5M KOH was added gradually at 50°C under stirring. After 10 minutes, 0.5 ml glutaraldehyde was added with continuous stirring for 30 minutes. Chitosan/magnetite particles were washed with 0.1 M Na-acetate buffer (pH 5.0), then 10 ml enzyme solution were added with stirring at 20°C for 30 min, then left at 4°C overnight. The particles were washed, stored at 4°C in 0.1 M Na- citrate buffer (pH 5.0). The enzymes activity and protein content were determined.

Encapsulation using Ca-alginate

A methodology of the encapsulated enzymes adapted by Viet *et al.* (2013) was followed. Ten ml of enzyme solution were mixed with 40 ml Na-alginate (2%). The mixture was then extruded using a disposable syringe in 2% $CaCl_2$ solution at 3 cm distance above the gelling solution. After 30 min, the beads were separated by filtration, washed three times with sterile distilled water then the enzymes activity and protein content were determined.

Electron microscopy detection of immobilizeenzymes

Enzymes immobilized by cross-linking and encapsulation were detected by scanning electron microscope JEOL, JSM-5400 LV (SEM) unit, Assiut University, Assiut, Egypt. While, immobilized enzyme on chitosan-coated magnetic nanoparticles (Fe_3O_4-CS) was detected by transmission electron microscope (TEM).

Effect of temperature and pH on the immobilized enzyme

Optimum temperature

To determine the optimum temperature for enzyme activity immobilized by different methods, 0.05 g filter paper (for FPase), 0.9 ml carboxymethyl cellulose (for CMCase) or cellobiose (for β-glucosidase) were separately incubated with 0.2 g immobilized enzyme in 1 ml citrate buffer at different temperatures (30, 40, 50, 60, 70 and 80°C) for 30 min (Viet *et al.*, 2013). The activity of the enzyme was assessed.

Optimum pH

The activities of the three enzymes (FPase, CMCase and β-glucosidase) were determined by adding the enzyme substrates into 50 mM Na-citrate buffer and different pH values (pH 3.5, 4, 4.5, 4.8, 5, 5.3 5.5, 6, 6.5, 7) were adjusted then, the mixture was incubated with 0.2 g immobilized enzyme in 1 ml citrate buffer at 50°C for 30 min (Yin *et al.*, 2013).

Reusability

To evaluate the reusability of the immobilized enzymes resulted from different methods, 0.2 g of immobilized enzyme was added to enzyme substrate adjusted at the pH and incubated at optimum temperature. After the first reaction, the immobilized enzyme was washed with Na-citrate buffer and the activity was measured. The experiment was repeated

and the activity in each time was measured (Rahim *et al*. 2013).

Enzyme stability during storage

Among the immobilized cellulases, encapsulated enzyme recorded the highest activity, so that, it was stored at 4°C in Na-citrate buffer (pH 5.3) for 5 weeks. The enzyme activity was weekly evaluated (Yin *et al*. 2013).

Relative activity of immobilized cellulases (in relation to free enzyme) =

$$\frac{\text{activity of immobilized enzyme}}{\text{activity of free enzyme}} \times 100$$

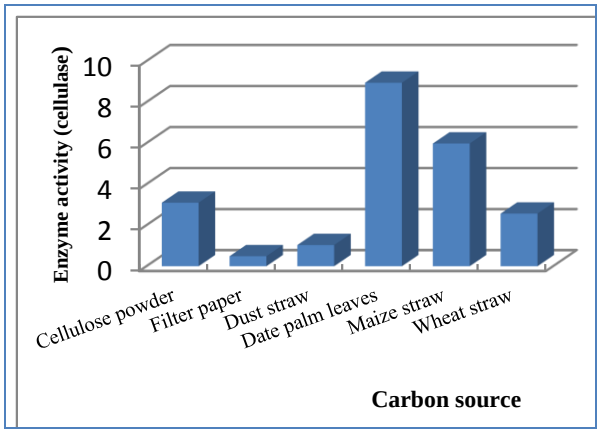
Relative activity of optimum immobilized cellulases =

$$\frac{\text{activity at different parameters (pH, temperature, ... ect)}}{\text{activity at optimum degree of the same parameter}} \text{X } 100$$

RESULTS

Optimization of conditions for cellulolytic enzymes

The results revealed that maximum enzyme production by *P. brevicompactum* was achieved in a medium with date palm leaves as a sole carbon source and sodium nitrate as a sole nitrogen source and initial pH 6, incubated at 30°C for 9 days (Figs. 1 and 2).



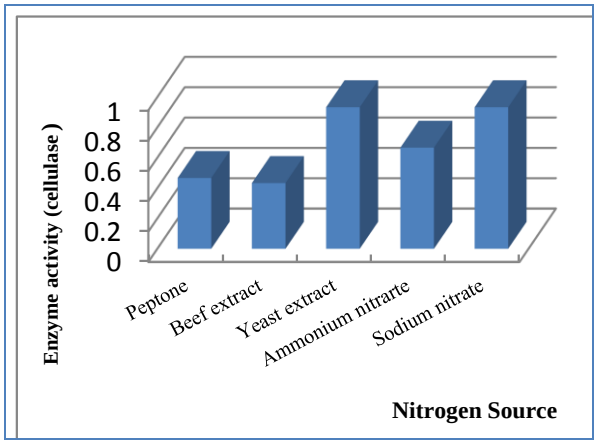


Figure (1): Effect of different carbon and nitrogen sources on cellulases production by *P. brevicompactum* AUMC 10987 in liquid medium.

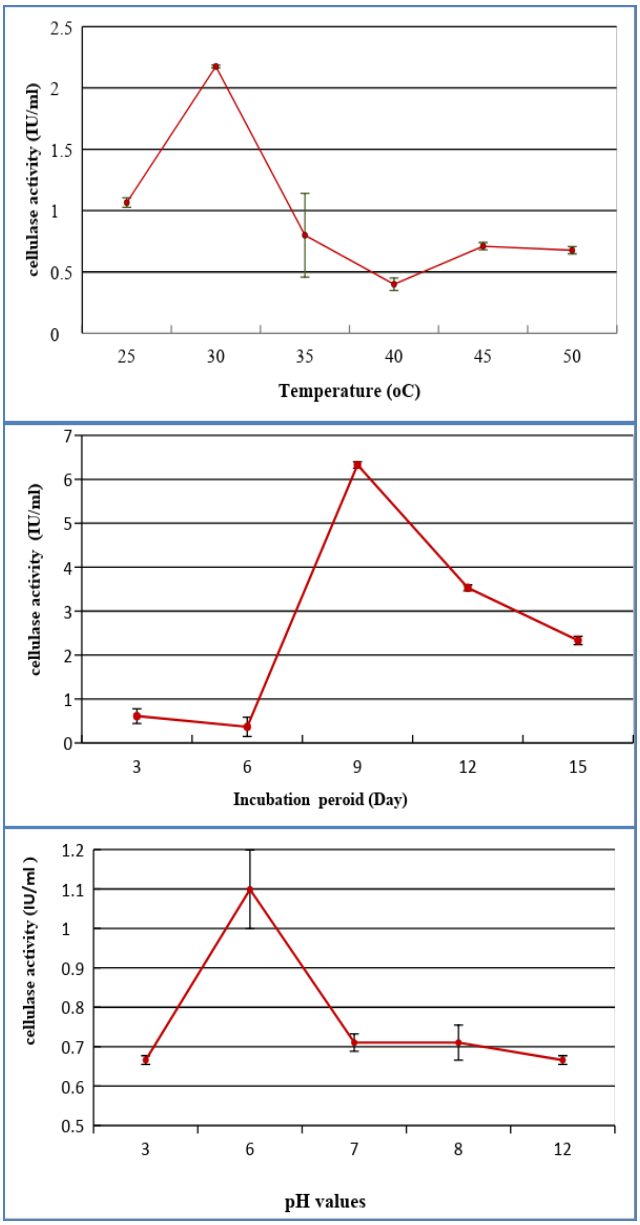


Figure (2): Effect of different incubation temperatures (a), incubation periods (b) and initial pH values (c) on cellulases produced by *P. brevicompactum* AUMC 10987 in liquid medium.

Enzyme production in submerged and solid state fermentations

The results showed that the activities of different enzymes (FPase, CMCase and β-Glucosidase) as well

as, protein content were respectively 3.5, 7.27 17.66 IU/ml/min, 154.7 µg/ml in solid state fermentation (SSF); while in submerged fermentation (SF) were 2.55, 5.86, 12.89 IU/ml/min and 57.7 µg/ml (Fig. **3**).

Activities of precipitated cellulases

Generally, partial purification of enzymes improved their activities, either by precipitation (FPase, 8.07; CMCase, 18.26 and β-Glucosidase, 48.25 IU/ml/min), n-propanol precipitation (5.66, 14.66 and 35.87 IU/ml/min) or acetone precipitation (4.22, 12.25 and 27.32 IU/ml/min)

(Fig. **4**). Moreover, protein content was increased after precipitation (214.4, 1997.7 and 156.03 µg/ml by the three precipitation methods respectively.

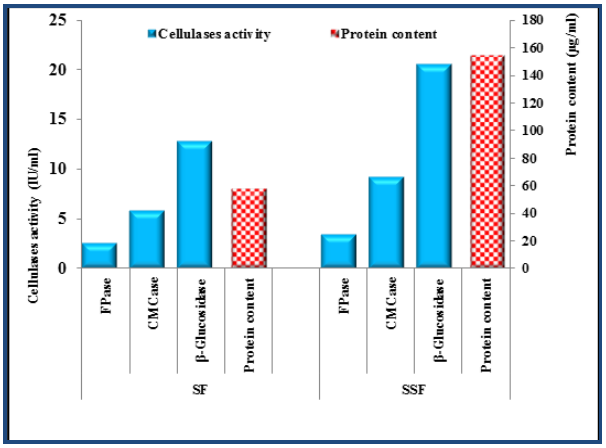


Figure (3): Cellulases activities and protein content produced by *P. brevicompactum* AUMC 10987 in submerged and solid state fermentations.

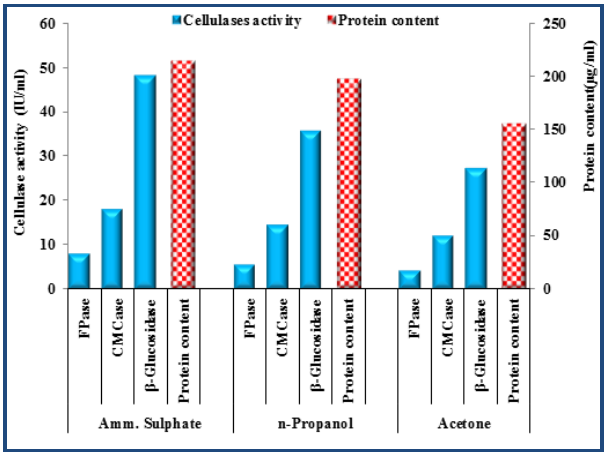


Figure (4): Cellulases activities and protein content measured after precipitation of enzyme by ammonium sulphate, n-prpanol and acetone.

Effect of temperature and pH on partially purified enzyme

Among different temperatures (30-80°C) and pH (3.5-7) tested, the precipitated enzyme exhibited their maximum activity at 50℃ and pH 4.8 (Supplementary S1)

Immobilization of cellulases by different methods and their effects on enzyme activities

Cross-linking using glutaraldehyde

It was observed that, under scanning electron microscope (SEM), glutaraldehyde links with amino groups in enzyme molecules forming cross-linked immobilized cellulases with matrix shape (Fig. 5a). It is worthy to mention that, cross-linking retained about 71.4, 73 and 75.61% relative enzyme activities for FPase, CMCase and β-glucosidase respectively.

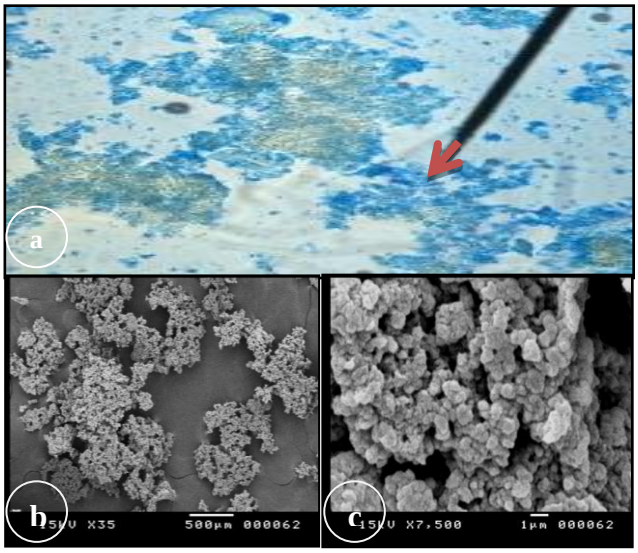


Figure (5a) Morphology of cross-linking immobilized cellulases under light microscope (a) and under *scanning electron microscope* (b, c).

Magnetic nannocarriers

The immobilization of cellulases on magnetic nannocariers required coated material such as chitosan which is added during immobilization procedures, using chitosan enzyme molecules that aggregate around nanoparticles formed chitosan-enzyme coated magnetic nanoparticles (CH-MNPs) (Fig. 5b). In comparison with the free enzymes, higher relative activities (79.1%, 80.61% and 83.48%) were obtained for FPase, CMCase and β-glucosidase respectively.

Encapsulation using Ca-alginate

The beads resulted from encapsulation of the enzyme by Ca-alginate were characterized by irregular shapes, 900-1000 µm size (Fig. 5c). The encapsulated FPase, CMCase and β-glucosidase had high relative activities of 81.78, 82.75 and 84.62% respectively

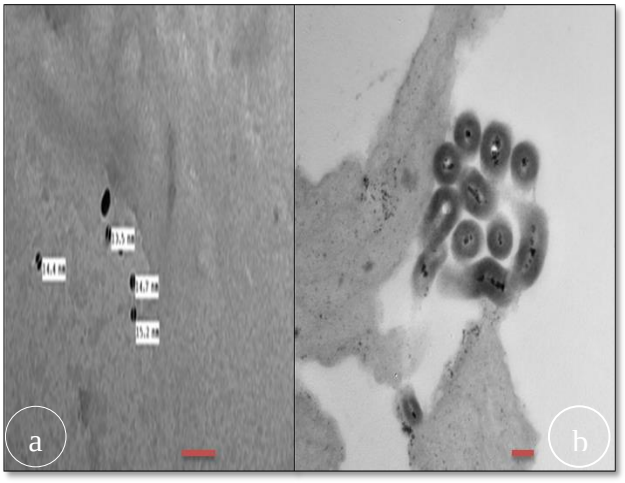


Figure (5b) Transmission electron microscopy (TEM) images of (a) Iron oxide nanoparticles (IONPs) (Fe_3O_4 + Fe_2O_3, diameter in mean = 15.2 ± 2.6 nm), and (b) CH-MNPs immobilized cellulase. Where the scale bar = 100 nm.

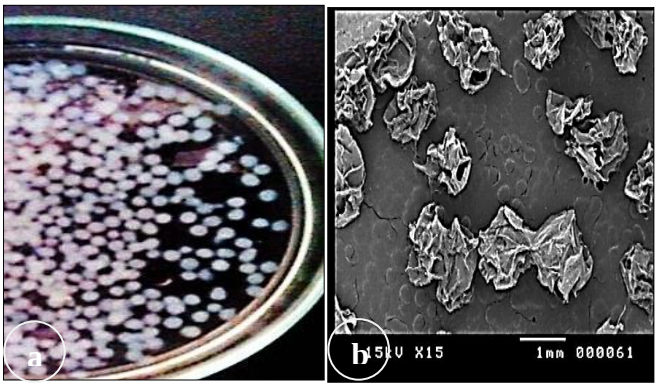


Figure (5c) Beads of immobilized cellulases encapsulated by Ca-alginate in petri dish (a) and under scanning electron microscope (b, c).

Optimization of some parameters for immobilized enzymes

Temperature

The maximum activity of FPase and CMCase immobilized by cross-linking reached 5.76 and 13.33 IU/ml/min respectively at 50°C (Supplementary S2a). However, the optimum temperature for β-glucosidase, which gave 36.48 IU/ml/min, was 60°C. Also, enzyme immobilized by magnetic nannocarriers gave the best activity at 60°C (Supplementary S2b). Immobilization on Ca-alginate maintained maximum enzyme activities at 50°C (6.65, 15.11 and 40.83 IU/ml/min for FPase, CMCase and β-glucosidase respectively) and these immobilized enzymes showed thermostability up to 80°C ((Supplementary S2c).

Optimum pH

The results showed that, from different pH values tested (4-7), pH 6 yielded the highest cross- linking immobilized enzyme activities (3.91, 15.74 and 40.04 IU/ml/min) for FPase, CMCase and β-glucosidase respectively (Supplementary S3a). The maximum enzyme activities for FPase, CMCase and *β*-Glucosidase (4.33, 17.86 and 38.04 respectively) immobilized by magnetic nannocarriers were obtained at pH 6 (Supplementary S3b).

Using Ca-alginate for cellulase encapsulation, the maximum activities (4.43, 18.56 and 45.07 IU/ml/min forFPase, CMCase and β-glucosidase respectively) were shown at pH 5.5 (Supplementary S3a).

Enzyme reusability

Results in Figure (6a) revealed that, the cross-linking immobilized enzyme could be reused twice after the main reaction, whereas after 8 hours, the immobilized enzyme activities decreased to 51.55, 64.58 and 75.38% for FPase, CMCase and β-glucosidase respectively, while after that, the immobilized enzyme lost its activity. It was found that immobilization by magnetic nannocarriers was the ideal technique to maintain stability and reusability of the immobilized enzyme forup to five consecutive cycles at 60°C, whereas, the immobilized β-glucosidase maintained about 67% while, FPase and CMCase maintained about 62.5% and 41.59 % of their activities respectively, after the third cycle (Fig. 6b). The *β*-glucosidase in Ca-alginate entrapped enzyme showed 13.33% relative activity after four cycles (Fig. 6c).

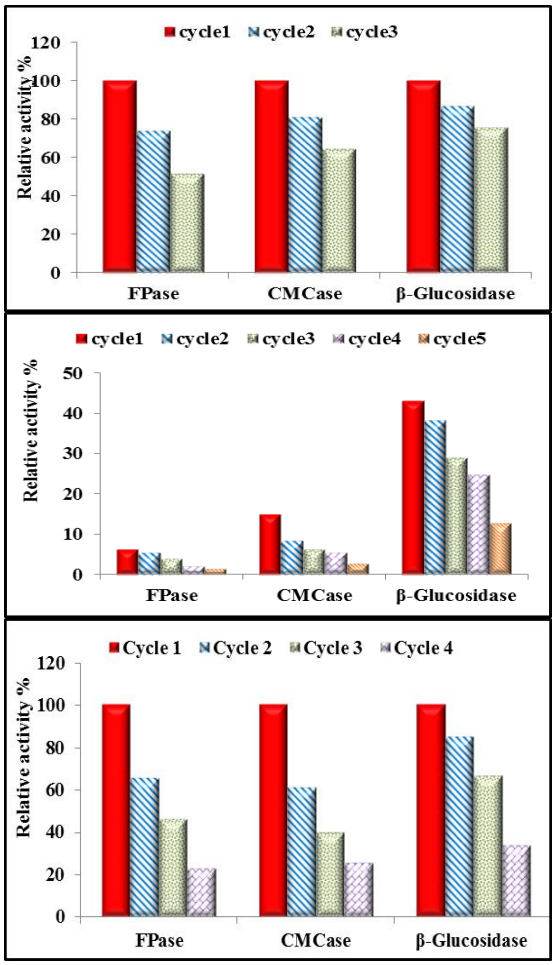


Figure (6): Reusability of (a) cross-linking, (b) magnetic nannocarriers, and (c) Ca-alginate encapsulated immobilized enzymes.

The influence of storage periods on the activity of CMCase immobilized in Ca-alginate

From the standpoint of preference of Ca-alginate as immobilizing agent, beads of enzyme immobilized in Ca-alginate were stored in Na-citrate buffer (pH 5.3) at 4°C for 5 weeks and the activity was measured every week. The obtained results revealed that the relative activity was reduced during storage from 100% to 18.66 % at the end of storage period (after 5 weeks) (Table **1**).

Table (1): Relative activity of CMCase enzyme immobilized by encapsulation at different storage periods

Storage Periods / weeks	% Relative activity
0	100
1	80.74
2	56.25
3	42.36
4	20.65
5	18.66

DISCUSSION

The maximum amount of endo-β-1,4-glucanase enzyme was produced in the presence of sodium or calcium nitrate as nitrogen sources, after 8 days of incubation at 30°C with pH 6 of *Alternaria alternata*, *A. citri*, *Cochliobolus spicifer* and *C. globosum* (El-said *et al.* 2014). Also, sodium nitrate was found to be the best nitrogen source for cellulase production by *Aspergillus fumigatus* (Sherief *et al*. 2010). Some other studies showed also that 30°C and pH 6 were the optimum conditions for cellulase production by some fungal species such as *Trichoderma atroviride* (Bairagi 2016) and *Penicillium* sp. (Prasanna *et al*. 2016). In India, Padmavathi *et al*. (2012) and Isaac and Abu-Tahon (2015) found that, maize straw and corn stover were the best carbon sources used for FPase, CMCase and β-glucosidase production by *A. terreus* and *Mucor plumbeus* among the other lignocellulosic tested materials.

However, in both fermentation methods, β-glucosidase showed the highest activity among the three enzymes tested. This may be attributed to the less complexity and hence easy assimilation of the substrate by the fungal isolate. In this respect, SSF for the production of cellulases offers numerous advantages such as high productivity, relatively high concentrations of the products and less effluent generation (Acharya and Chaudhary 2012) as well as, higher yields and product stability (Singhania *et al*. 2009).

It seems that different precipitants constructed a more stable structure for the enzyme and keep the enzyme in its origin conformational even after immobilization (Sulaiman *et al*. 2014). In consistence with our results, Kalyani *et al*. (2015) reported that the activity of partially purified enzyme by ammonium sulphate increased than that of the crude enzyme. Therefore, ammonium sulphate has been the most widely precipitant used in protein separation and purification among many other salts because of its high solubility and is relatively inexpensive (Duong-Ly and Gabelli 2014).

Our results were in harmony with the findings of Xu *et al*. (2015) and Zhang *et al*. (2016). pH 5 was also reported as optimum pH for maximum enzyme activities of *A. niger* and *A. fumigates* (Immanuel *et al*. 2007), while, pH 4.5 gave the maximum activity of free cellulose in the study of Xu *et al*. (2015).

The slight decline in activity of cross-linked immobilized cellulase was also reported by Sheldon (2007). This may be due to the reaction of cross-linker with amino acid residues that are crucial for the activity of the enzymes or when the concentration of glutaraldehyde solution was too much, lead the enzyme molecules to form multi-point binding with the carrier and increase the amount of the enzyme bound to active aldehyde may change the spatial structure of the active center of enzyme (Chen *et al*. 2013). Cross-linking technique utilizes a bi- or multifunctional compound to bind with the functional groups of the enzyme to form insoluble cross-linked complex, whereas, the enzyme molecules are covalently bounded to each other to create a matrix. Successful preparation of cross-linked enzyme aggregates (CLEAs) from a broad range of enzymes was obtained (Šulek *et al*. 2011).

The immobilization using iron oxide nanoparticles, enhanced the biocatalytic properties of an enzyme, including stability and reusability can provide higher surface area for high loading of enzyme (García *et al*. 2014), this may explain the relatively higher enzyme activities than that obtained by cross-linking. Recently, ferric oxide (Fe_3O_4) magnetic nanoparticle have been paid much attention by several researchers for immobilization of cellulases, due to its non or at least low toxicity, good biocompatibility, very good candidate, without residual magnetism, with simple, quick and low-cost collection of enzymes from a complex mixture with an external magnetic field, high enzyme loading capability, due to their large specific surface area and lower diffusion limitation in solution (Li *et al*. 2013; Tang *et al*. 2014).

Cellulases encapsulated by Ca-alginate retained up to 84.62% of their activities, similar results were obtained by Viet *et al.* (2013). Enzyme encapsulated within alginate beads is preferred due to easy for formulation, mild gelation conditions, non-toxic, biocompatibility, low cost and resistance to microbial attack (Won *et al.*, 2005; Quiroga *et al*. 2011). Encapsulation is the process by which the enzyme is enclosed physically or chemically within semi-permeable membrane. Attaching or entrapping the protein in support materials can prevent the enzyme from leaving while allowing substrates, products, and co-factors to permeate to the enzyme (Martinek and Mozhaev 1985). Calcium alginate is used extensively because it provides stability and biochemically inert (Abd El-Ghaffar and Hashem 2013; Viet *et al*. 2013).

Based on our results, it was found that cross-linked cellulase immobilized by chitosan and glutaraldehyde had optimum temperature at 50°C (Podrepšek *et al*. 2012; Yu *et al*. 2012; Li *et al*. 2015). Also, during studying on immobilized cellulases activities retained 70% of the maximum activity at 50°C in the study of Dal Magro *et al*. (2016). However, in the current study, the optimum temperature for β-glucosidase, which gave 36.48 IU/ml/min, was 60°C. Also, enzyme immobilized by magnetic nannocarriers gave the best activity at 60°C. This was in accordance to the findings of Abraham *et al.* (2014) on immobilization using cellulase complex binding onto magnetic nanoparticle. It was reported that the immobilization of the enzyme on the solid support increased the thermostability of the enzyme which can tolerate higher temperature whereas the free enzyme that denature at high temperature (Kumar *et al.* 2018)

In respect to our results, Chang and Juang (2005) reported the same optimum temperature of encapsulated cellulases. It was suggested that, decreasing the enzyme activities above 50^{o}C may be related to the vibration and movement of the enzyme molecule which can affect the hydrogen bonding in the enzymes structure. Therefore, this may reduce the catalytic power of enzymes and

lastly tend to denaturate the enzymes (Mat Radzi *et al.* 2005; Lee *et al*. 2008; Abd Rahim *et al*. 2013).

Laing *et al.* (2006) found that the optimal pH of cross-linked urease was 6.5. The maximum enzyme activities for FPase, CMCase and *β*-Glucosidase (4.33, 17.86 and 38.04 respectively) immobilized by magnetic nannocarriers were obtained at pH 6. Similar findings were also obtained by Choi *et al.* (2009) and Zahoor *et al*. (2011).

Almost our results are similar to the finding of Keerti *et al*. (2014) for the immobilized *β*-glucosidase in alginate gel which had the highest activity at pH 5.0. It was deduced that, the surfaces of the beads which localize the enzymes have a cationic and anionic nature that will produce microenvironment charges and then it will affect the nature of active enzyme. So, the increasing or decreasing of pH from the optimum value could reduce the activity of enzymes due to charge acquired by the support (Abd Rahim *et al*. 2013).

One of the main targets for immobilization is the reuse of the enzyme to reduce cost for industrial process. In this respect, Khorshidi *et al.* (2016) reported that the CMCase activity decreased sharply to 30% of initial activity after two cycles of hydrolysis, however retained this level up to 6 cycles. Reuse of immobilized enzyme for more than one cycle may be attributed to multipoint, multi-subunit immobilization or generation of favorable environments (Xu *et al*. 2014), but decreasing the activity could be related to the denaturation and the leakage of enzyme upon use and diffusional effects (Ye *et al*. 2006). It is worthy to mention that, our results were relatively higher than that obtained by Abraham *et al*. (2014) who found that the immobilized enzyme retained 50% of its activity after four cycles. In accordance, Keerti *et al*. (2014) mentioned that the immobilized *β*-glucosidase in alginate was reused 4 times, with gradual decrease in the residual activity to 17.85%.

Our results were relatively more valuable than the finding of Keerti *et al.* (2014) who, reported that the immobilized cellulases in alginate retained about 17.74% of its original activity at 4◦C after 25 days. However, Yin *et al.* (2013) found that the immobilized cellulase showed a gradual decrease in activity and lost 14% of initial activity after 4 weeks.

Conclusion

Immobilized enzyme has superiority over free enzyme in different biotechnological processes, due to its high stability and low cost. The current study could provide a robust and highly valuable immobilized enzyme for different industrial and biotechnological applications.

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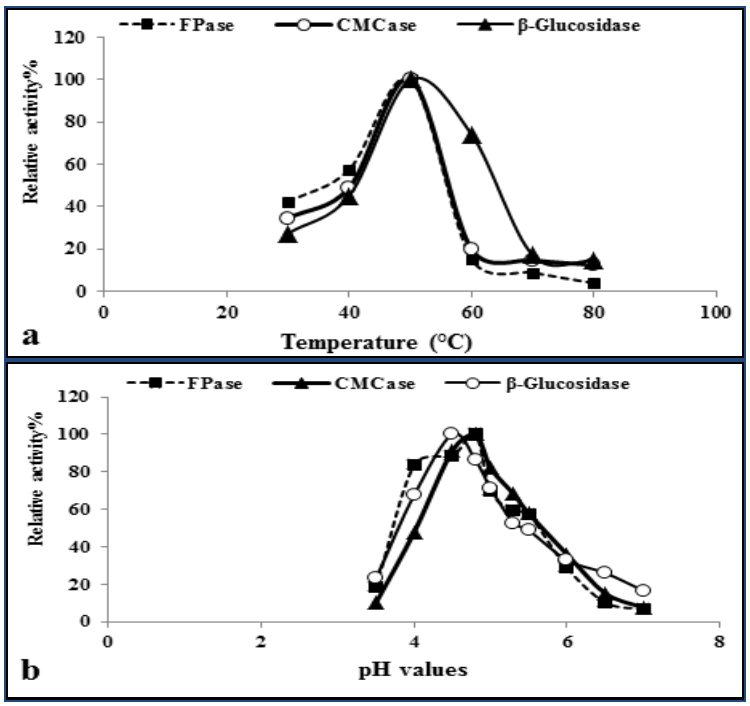
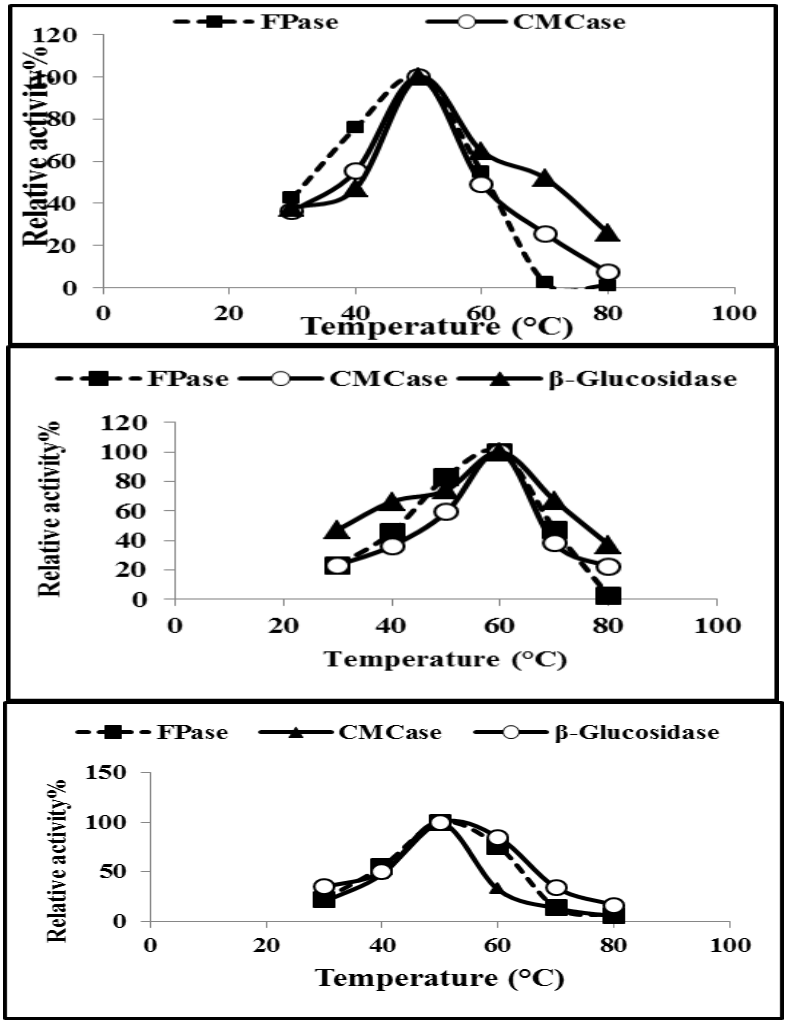
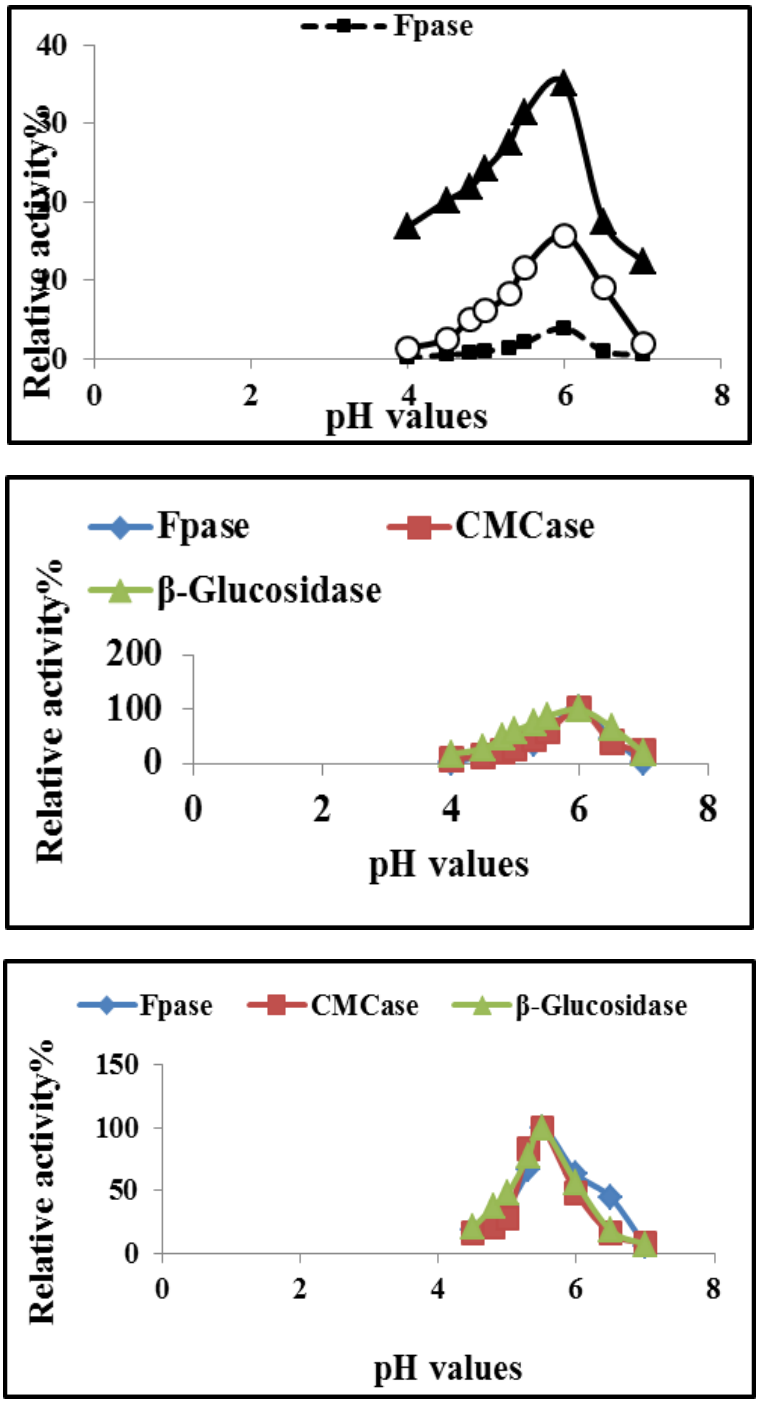


Supplementary (S1): Relative activities of precipitated cellulases (FPase, CMCase and β-glucosidase) produced by *P. brevicompactum* at different temperature degrees (a) and pH values (b).



Supplementary (S2): Relative activities of cellulases (FPase, CMCase and β-glucosidase) immobilized by cross-linking (a), magnetic nannocarrier (b), and Ca alginate encapsulation (c) at different temperature degrees.



Supplementary (S3) Relative activities of cellulases (FPase, CMCase and β-glucosidase) immobilized by cross-linking (a), magnetic nannocarrier (b), and Ca-alginate (c) at different pH values.

تثبيت انزيمات السلولاز المنتجة بواسطة Penicillium brevicompactum AUMC 10987 ، باستخدام Cross-Linkage، Chitosan-Coating وEncapsulation

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الملخص العربي

صُممت هذه الدراسة لتحسين الظروف المختلفة وذالك لتحسين إنتاج الإنزيم بواسطة Penicillium brevicompactum AUMC 10987 ، وترسيب وتثبيت انزيمات السلولاز عبر أسلوب الربط المتقاطع باستخدام glutaraldehyde ، و nanocarriersالمغناطيسي وتغليف باستخدام .Ca-alginate تم تحقيق الحد الأقصى من نشاط السلولاز في أوراق نخيل التمر متوسطة الحجم ، نترات الصوديوم ، بعد حضانة تسعه أيام عند 30 درجة مئوية ودرجة الحموضة 6. كان تخمر الحالة الصلبة طريقة أكثر ملاءمة في إنتاج سلولاز. بعد ترسيب أملاح كبريتات الأمونيوم ، تجاوزت أنشطة الإنزيم مرتين ، مما أدى إلى بناء المزيد من الثبات لهيكل الإنزيم وإعطاء أقصى قدر من الأنشطة عند 50 °C و 4.8 درجة الحموضة. وبالتالي ، يمكن أن يعمل الإنزيم المرسب في ظروف درجة حرارة وحموضة أعلى. كان عدم الحركة من cellulases بواسطة التغليف التقنية الأكثر قيمة الاحتفاظ نشاط الانزيم. علاوة على ذلك ، ظل الإنزيم الثابت نشطًا حتى 80 درجة مئوية ، مما أعطى أقصى نشاط له عند 50 درجة مئوية و 5.5 درجة حموضة. ويفضل أنزيم مغلف داخل حبيبات الجينات بسبب سهولة التركيب ، وظروف الهلام الخفيفة ، وعدم السمية ، والتوافق مع الحياة ، وانخفاض التكلفة ومقاومة الهجوم الميكروبي. كان nanocariers المغناطيسي يجمد فرصة لإعادة استخدامها بعد 4 دورات / 16 ساعة. يمكن أن توفر الدراسة الحالية إنزيم ثابت قوي وقيمة لتطبيقات صناعية وبيوتكنولوجية مختلفة.

كلمات البحث: Ca-alginate، Cellulase، Encapsulation، Penicillium brevicompactum AUMC 10987