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Production, Characterization and Immobilization of a *Fusarium solani* Lipase by Chitosan Magnetic Nanoparticles

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LIPASE producing fungus was isolated and identified as a strain of *Fusarium solani* based on its 18s rDNA sequence. The enzyme it produces was purified by diethyl amino ethyl sephadex (DEAE-sephadex) column chromatography. The specific activity of the pure enzyme was 1.98 U/mg protein. The kinetics study showed that K_m and V_{max} values were 0.63µM and 29.4µM/min/mg protein, respectively. The MW was 95.27 kDa. Effects of pH, incubation temperature and organic solvents on the lipase activity were studied. The maximum enzyme activity was obtained at pH 8.5 and incubation temperature 35°C. Hexane and butanol inhibited enzyme activity by 51% and 72.6 %, respectively, while DMSO stimulated the activity by 47.8%. The lipase was immobilized by fusion to chitosan-coated iron oxide magnetic nanoparticles and cross-linked by glutaraldehyde. The reusability and storage period of the immobilized enzyme showed that the enzyme retained 80% of its activity after 15 reuse cycles and retained 97% of activity after 30 days of storage at 4°C. The immobilized lipase was tested for synthesis of sugars-oleate esters and the ester products were analyzed by liquid chromatography tandem-mass spectrometry (LC/MS/MS). This investigation identified the potential for use of the obtained *F. solani* lipase in industrial applications that utilize organic solvents or alkaline pH values, such as detergent industry.

Keywords: *Fusarium solani*, Lipases, Magnetic nanoparticles, Enzyme immobilization, Chitosan.

Lipases (EC 3.1.1.3) catalyze the hydrolysis of glycerides to free fatty acids, and glycerol. Due to their large number of applications, several studies have been conducted for isolation of lipase-producing microorganisms with various characters such as resistance to a wide range of temperatures, organic solvents, and to acidic and alkaline pH. Lipases are used in pharmaceutical formulations such as cosmetics and to produce various intermediates used in manufacture of medicine (Rohit *et al.*, 2001), as a biosensor and as a diagnostic tool (Pandey *et al.*, 1999; Lott & Lu, 1991 and Higaki *et al.*, 2000). In the food industry lipases behave as a

flavoring agent in dairy products, bakery, beverages, and meat and fish (Saxena, *et al.*, 1999; Reetz, 2002 and Macedo *et al.*, 2003). Lipases are used in detergents (Bajpai & Tyagi, 2007 and Weerasooriya & Kumarasinghe, 2012) and have environmental applications to hydrolyze oils and grasses (Pandey *et al.*, 1999 and Lin *et al.*, 2012) and for organic synthesis of esters that have a variety of applications (Berglund & Hutt, 2000)

Immobilized enzymes increase enzyme stability at various pH values, temperatures and ionic strengths. Furthermore, immobilized enzymes can be recycled from the reaction mixture. Magnetic nanoparticles have several advantages as serving as the supporting material for immobilized enzymes over competing materials, providing a higher surface area that allows for greater enzyme loading, and by enabling separation from the reaction mixture by application of a magnetic field (Johnson *et al*., 2011).

This study aimed to isolate a lipase producing fungus and to characterize the enzyme by studying the effect of pH, temperature, organic solvents, reusability and enzyme kinetics, as well as immobilization by chitosan-magnetite nanoparticles and application in sugar-ester synthesis.

Materials and Methods

Isolation of lipase producing fungi

Rhodamine-B agar medium was used for isolation of lipase producing fungi by a selective plating technique (Rajendiran *et al.*, 2011).

Lipase assay

Lipase activity was determined using *p*-nitrophenyl palmitate (*p*-NPP) (MW 377.52) (Sigma) as a substrate (Kantak *et al.*, 2011)

Fungus identification

The fungal isolates were identified by using 18S-rDNA sequencing (Manoj *et al.*, 2014). The 18S rDNA sequence of a purified strain was amplified by PCR with forward primer 5′-CCTGGTTGATCCTGCCAG-3′ and reverse primer 5′-TTGATCCTTCTGCAGGTTCA- 3′. The PCR reactions were carried out as follows: one initial cycle at 95°C (5 min), followed by 34 cycles of 95°C (1 min), annealing at 55°C (1 min), 72 °C (1.5 min) and ended with incubation at 72°C for 10 min. The amplified product was electrophoresed on a 1.0% agarose gel. The fragment of interest was excised from the gel and purified with Rapid Recovery Kit (Gel), followed by sequencing (Sangon Shanghai, China). Sequence alignment of the 18S rRNA gene sequence with other sources in Genbank was performed by using the BLAST function at NCBI website (http://www.ncbi.nlm.nih.gov/) and then a phylogenetic tree was constructed with MEGA 3.1 software.

Lipase production and purification

A disk from a five days old slant of *F. solani* that had been grown on potato dextrose agar was used to inoculate 250 ml conical flasks containing 50 ml of the

following production medium (g/l): Olive oil: 10, Peptone: 1, K_2HPO_4: 1.0, $MgSO_4.7H_2O$: 0.3, $CaCl_2$: 0.25, $ZnSO_4 \cdot 7H_2O$: 0.03, $FeSO_4 \cdot 7H_2O$: 0.025, $MnSO_4 \cdot 7H_2O$: 0.015. The flasks were incubated for five days on a rotary shaker at 28±2°C and 150 rpm. The enzyme was precipitated from the supernatant by stepwise addition of 150 ml of cooled acetone to 100 ml of the supernatant under continuous stirring. The enzyme precipitate was separated by centrifugation at 8000 rpm for 15 min under cooling. The precipitate was dissolved 0.02M sodium phosphate buffer (pH 6.6). The enzyme was purified using DEAE-sephadex (Sigma) column stepwise eluted by 5 ml portions of a mixture of phosphate buffer (pH 6.6): NaCl (100: 0), (95:5), up to (0: 100%). Thirty eight fractions were collected. Soluble protein was determined by Lowry method (Lowry *et al.,* 1951) and the specific activity was calculated as U/mg protein.

MW determination

The molecular weight of purified lipase enzyme was determined by SDS-PAGE. The gel image was analyzed by gel documentation software (Alpha Ease FC 4.0).

Enzymes kinetics study

The Michaelis–constant and maximum velocity (K_m and V_{max}) of the purified lipases were determined by using various concentrations of *p*-nitrophenyl palmitate as substrate from 0.2 mg/100µL to 1.2 mg/100µL. The values of kinetic parameters, K_m and V_{max} were determined from a Lineweaver–Burk plot.

Effect of pH and temperature on the lipase stability

Buffer solutions (50mM) of different pH values were used which includes citrate buffer (4-5), phosphate buffer (6-7) and Tris-HCl buffer (8-9). The purified enzyme was incubated for 30 min in buffers with varying pH values at 35^{o}C and then the enzyme activity was determined. To study the effect of temperature, the purified enzyme solution in phosphate buffer (50mM) pH 6.6, was incubated for 30 min at 5, 15, 25, 35, 45, 55 and 65 ^{o}C followed by determination of the enzyme activity.

Effect of organic solvents

Equal volumes of substrate solution and one of the tested organic solvents (DMSO, acetone, acetonitrile, ethanol, iso-propanol, ethyl acetate, methanol, butanol or hexane) were mixed and left for 30 min at 35 ^{o}C before assaying for enzyme activity.

Preparation of magnetic nanoparticles based on iron oxide

The iron oxide magnetic nanoparticles (Fe_3O_4-MNPs) were prepared by co-precipitation of ferric chloride ($FeCl_3.6H_2O$,99%), ferrous chloride ($FeCl_2.4H_2O$, 98%) and aqueous ammonia (NH_4OH, 57.6 wt. %). 0.02M of ferric chloride and 0.01M of ferrous chloride were dissolved in 250 mL distilled water. The solution was heated at 60 ^{o}C with stirring and 50 ml of 0.01M NH_4OH was drop-wise added during heating. The solution was allowed to stand for 2 h. The separated

iron oxide magnetic nanoparticles were recovered by using a permanent magnet, washed two times with distilled water and lyophilized to obtain the final product (Kuo *et al.,* 2012).

Characterization of iron oxide magnetic nanoparticles

X-ray diffraction was performed to ensure the chemical composition of Fe_3O_4-MNPs, while the particle size were determined by JEOL JEM-2000 EX model scanning- electron microscope (SEM).

Preparation of chitosan-coated iron oxide magnetic nanoparticles

Chitosan was prepared from shrimp exoskeleton by the method described by Huang *et al.* (2004). Half g chitosan in 100 ml of 2% v/v acetic acid solution was mixed with 25 ml of 1 mg/ml sodium tripolyphosphate solution as a cross-linker to enhance colloidal stability, left for 10 min., followed by addition of 0.1 g of iron oxide magnetic nanoparticles (Fe_3O_4-MNPs) and vigorously stirred for 30 min. Fifty milliliter of 1 N NaOH was added slowly to the suspension to precipitate the chitosan coated iron oxide magnetic nanoparticles. The particles were recovered from the suspension by using a permanent magnet and washed with distilled water several times until the pH reached 7.0, then 10 ml of different concentrations ranging from 1% to 4% glutaraldehyde were added to the mixture and stirred for 2 h. The cross-linked nanoparticles were filtered and washed by phosphate buffer (0.05 M, pH 7.0) solution five times and stored in the refrigerator (4^{o}C) till later use (Kuo *et al.,* 2012).

Immobilization of lipases by chitosan-coated iron oxide magnetic nanoparticles

Ten ml of the chitosan-iron oxide nanoparticles (2mg/ml) were mixed with an equal volume of lipase in Tris-HCl buffer (0.05 M, pH 8.5) solution. The mixture was shaken at room temperature for 6 h. The immobilized lipases on chitosan-coated iron oxide magnetic nanoparticles were recovered by using a permanent magnet and washed five times by phosphate buffer solution (0.05 M, pH 7.0). The yield and efficiency of immobilized lipase was determined (Kuo *et al.,* 2012).

Reusability and storage stability of immobilized lipase

The activity of immobilized lipase was measured after up to twenty cycles of reuse. In order to investigate storage stability, the activity of both free and immobilized lipases were determined weekly during storage for 30 days at 4 ^{o}C.

Statistical analysis

The collected data were analyzed using ANOVA statistical analysis, by Design-Expert software version 7.0.0 (Camila *et al.,* 2011).

Results and Discussion

Identification of the most efficient fungal isolate

The most active fungal isolate was identified by cultural and morphological characteristics, and 18s rDNA sequence. According to BLAST result and

phylogenetic tree, the fungal isolate showed the most identity with *Fusarium solani* strain ZK004 with identity percent 98%. The topology of the resulting dendrogram (Fig. 1) showed that *Fusarium solani* strain ZK004 has high similar to the same cluster of *Fusarium falciform* & *Fusarium oxysporum*. The Blast searching showed that the strain of *Fusarium solani* ZK004 has more differences not only to the strain of *Fusarium incarnatum* from the database, but also with strain of *Gibberella moniliformis*, which is the name for the perfect stage of *Fusarium verticillioides*. Several previous investigations have been conducted for lipase production form *Fusarium* sp. (Maria *et al.*, 1999; Rafael *et al*., 2014 and Fernanda *et al*., 2015).

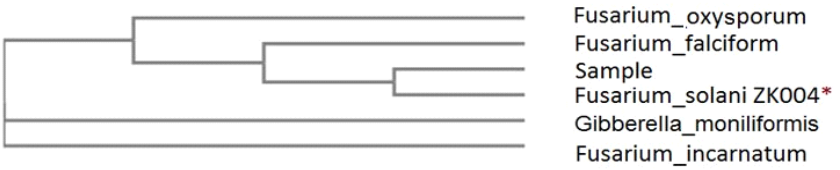


Fig. 1. Dendrogram after Clustal W2 multiple alignment of the 18srDNA sequences. (*) The tested strain obtained from NCBI Genbank by Blast searching .

Lipase purification

The lipase activity in the fugal supernatant was 23.2 U/ml. The enzyme activity after precipitation by acetone and DEAE-Sephadex purification were 21.1 and 17.1U/ml, respectively. These results reflect some loss in the enzyme during purification. However, the specific activity was increased after the Sephadex purification from 0.88 to 1.98 U/ mg protein. As shown in Fig. 2, the lipase is eluted in fraction number 27.

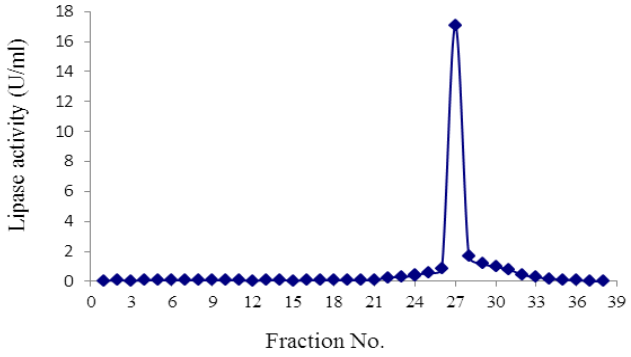


Fig. 2. Lipase activity in the fractions collected from DEAE-Sephadex column.

Lipase kinetics study

Lineweaver-Burk plot (Fig. 3) showed that K_m and V_{max} values were 0.63µM and 29.4µM, respectively.

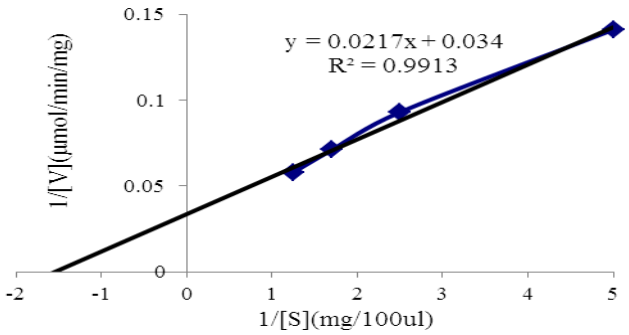


Fig. 3. Lineweaver-Burk plot for calculation of K_m and V_{max} values of *F. solani* lipase.

Molecular weight determination of lipase

The approximate molecular weight of the purified lipase enzyme is 95.27 kDa (Fig. 4).

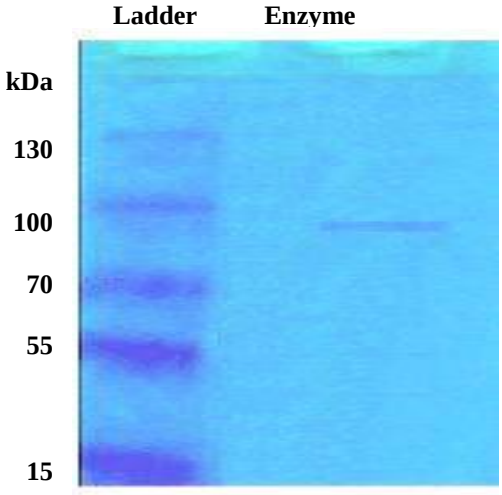
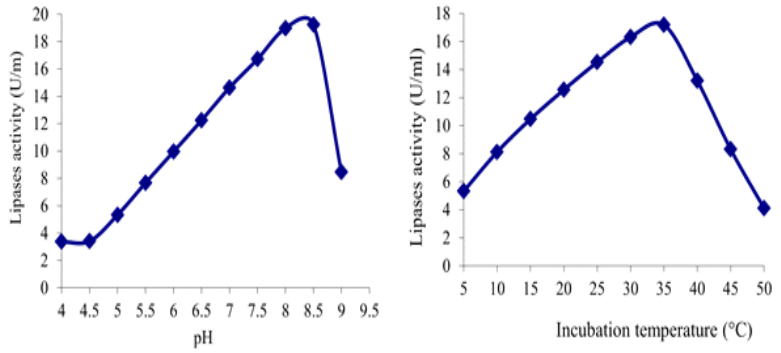


Fig. 4. SDS-PAGE showing the molecular weight of the purified lipase as compared with the protein ladder.

Effect of pH and temperature on the lipase stability

As shown in Fig. 5a, the maximum lipases activity was observed at pH 8.5. Therefore, we recommend use of this enzyme in detergents and alkaline applications. This result was in agreement with Kasana *et al.* (2008) and Amoozegar *et al.* (2008), where the enzyme was stable at pH range of 7.5-8.0 and retained 90% of its activity. On the other hand, maximum lipase activity was

obtained at 35 °C (Fig. 5b). Further increase in the incubation temperature inhibited the enzyme. Other alkaline lipases were previously isolated from *Fusarium globulosum* and *Rhizopus delemar* (Gulati *et al.*, 2005 and Haas *et al*., 2011).



(a) (b)

Fig. 5. Effect of pH and temperature on *F. solani* lipase stability.

Effect of organic solvents on the F. solani lipase activity

Stability against organic solvents is very important when using an enzyme in industrial applications such as synthesis of esters. The lipase activity was increased by 47.8% in presence of DMSO (dimethyl sulfoxide). Also, acetone, acetonitrile, iso-propanol, ethanol, methanol and ethyl acetate increases the enzyme activity (Fig. 6). This effect may be due to the solvent molecules interaction with hydrophobic amino acid residues present in the 'lid' that covers the catalytic site of the enzyme, thereby maintaining the lipase in a suitable conformation for the catalytic reaction. On the other hand, hexane and butanol inhibited the enzyme activity. These results were useful when using the obtained lipase for organic-synthesis reactions or other applications that include use of organic solvents (Doukyu & Ogino, 2010).

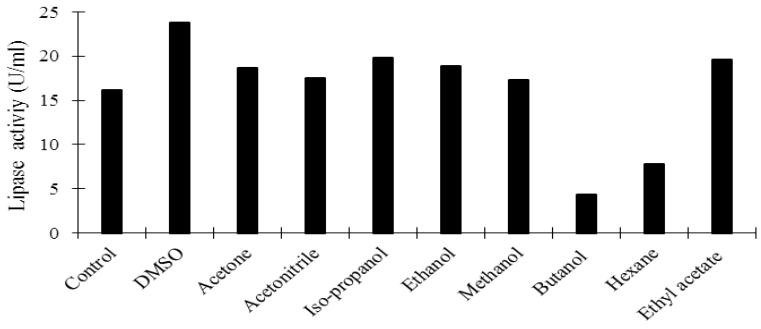


Fig. 6. Effect of organic solvents on the *F. solani* lipase activity.

Characterization of iron oxide magnetic nanoparticles (Fe_3O_4-MNPs)

X-ray diffraction verified the chemical composition of Fe_3O_4-MNPs. Scanning- electron microscopy described the size and shape of the iron oxide magnetic nanoparticles as rods with average length of 100 nm. (Fig. 7).

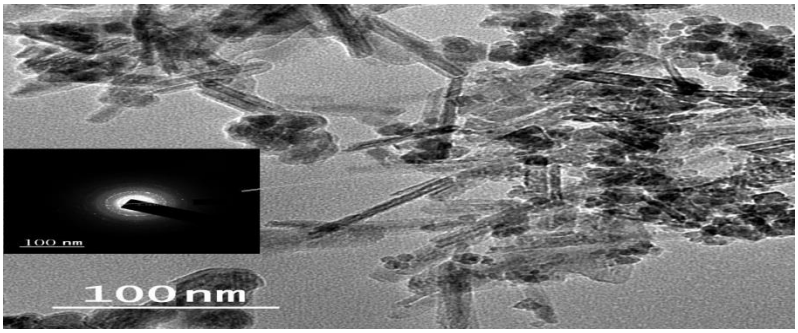


Fig. 7. SEM image for the iron oxide magnetic nanoparticles.

Reusability of the immobilized lipase

The reusability of the immobilized enzyme is one of the most important parameter that reflects how long this enzyme can be used, which is particularly interesting in terms of the economics of large-scale processes. The activity of the lipases immobilized onto chitosan-coated magnetic nanoparticles was measured after up to twenty cycles of reuse. After fifteen cycles of reuse, the lipases activity was retained up to 80% of the initial activity (Fig. 8).

The immobilization of *Mucor javanicus* lipases onto chrysotile (magnesium silicate) decreased the yield by 25% after its first reuse and by more than 70% after four reuse cycles (Silva & Jesus, 2003). On contrary, the covalent immobilization of *Rhizopus oryzae* lipases onto silica gel decreased the residual activity by only 20% after twenty reuse cycles (Lee *et al.*, 2006). The results obtained here were in agreement with Wang *et al.* (2010) who observed a slight decrease of enzyme activity (<15 %) after 12 cycles of reuse.

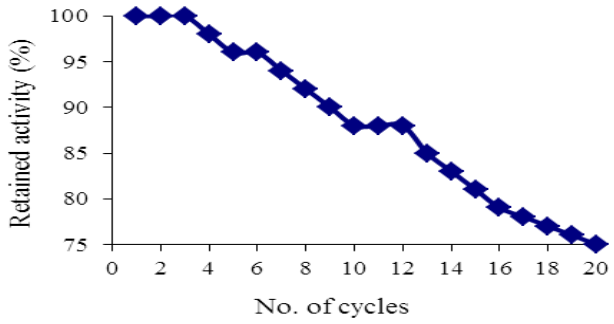


Fig. 8. Reusability of lipase immobilized onto chitosan-coated magnetic nanoparticles cross-linked by glutaraldehyde.

Storage stability of the immobilized lipases

The activity of free lipase decreased to 87% after four weeks; while the immobilized lipases decreased to only 97% after four weeks of storage at 4 ^{o}C. This result reflects the effect of the immobilization process in preserving enzyme activity.

Application of immobilized lipases for sugar ester synthesis

The use of lipase in organic synthesis (green synthesis) of esters is one of the most important applications. It was tested to synthesis sugar-esters of oleic acid. LC/MS/MS analysis of the ester products showed presence of mono and di-oleate glucose esters with molecular weight of 443.62 and 708.08, respectively. The molecular weights of mono oleate fructose and mannitol were 443.62 and 445.64, respectively (Fig. 9). Lipases have been used by many researchers for synthesis of sugar esters. Ferrer *et al.* (2005) utilized lipase from *Thermomyces lanuginosus* and *Candida antarctica* for synthesis of vinyl-sugars esters due to their antimicrobial activities. Lee *et al.* (2007) utilized lipase for synthesis of glucose-lauryl ester, while lipase of *Candida antarctica* was utilized by Jintian *et al.* (2012).

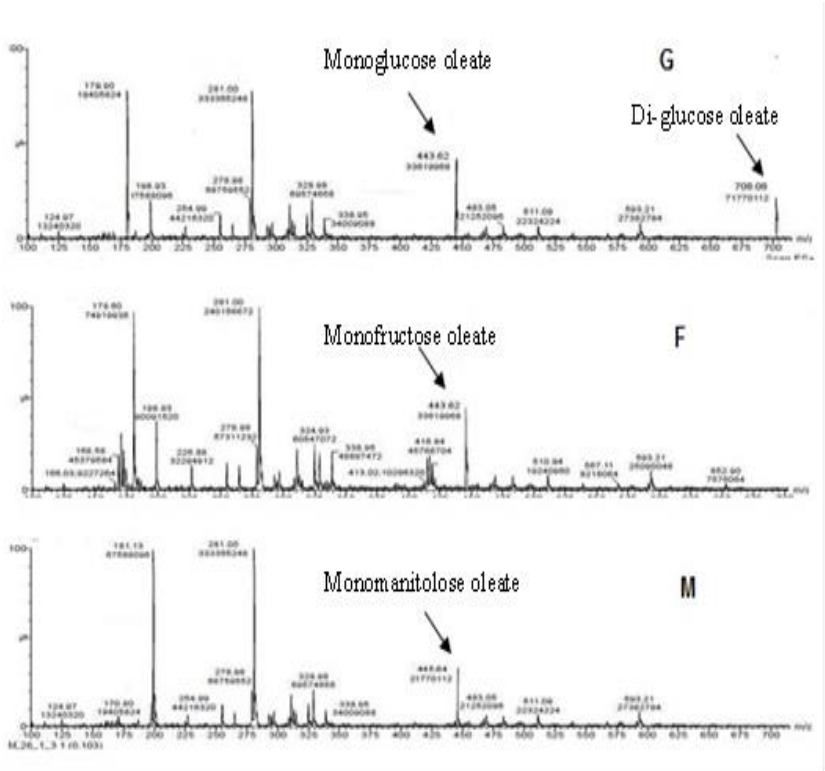


Fig. 9. LC/MS/MS spectra of the ester products synthesized by lipase with glucose (G), fructose (F) and mannitol (M).

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إنتاج و توصيف إنزيم الليبيز المنتج من *Fusarium solani* و تثبيته بواسطة حبيبات الكيتوزان المغلفة بأكسيد الحديد المغناطيسي بحجم النانو

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تم عزل أحد الفطريات المنتجة لإنزيم الليبيز و تعريفه عن طريق تتابع جين 18s-rDNA على أنه *Fusarium solani*. تم فصل و تنقية الإنزيم بإستخدام عمود الكروماتوجرافي الداي إيثيل أمينو إيثيل سيفادكس. وجد أن النشاط النوعي للإنزيم النقي يساوي ١.٩٨ وحدة نشاط إنزيمي لكل ملليجرام بروتين. دراسة حركيات الإنزيم أوضحت أن ثابت ميكائيلي و السرعة القصوى للإنزيم كانت ٠.٦٣ ميكرومول و ٢٩.٤ ميكرومول لكل دقيقة لكل ملليجرام بروتين على التوالي. الوزن الجزيئي للإنزيم ٩٥.٢٧ كيلو دالتون. تم دراسة تأثير درجة الحموضة و درجة حرارة التحضين و المذيبات العضوية على نشاط الإنزيم. تم الحصول على أعلى نشاط إنزيمي عند درجة حموضة ٨.٥ و درجة حرارة تحضين ٣٥°C. الهكسان و البيوتانول أظهروا تثبيط للنشاط الإنزيمي بنسبة ٥١٪ و٧٢.٦ ٪ على التوالي، بينما وجد أن الداي ميثيل سلفوكسيد حفز النشاط الإنزيمي بنسبة ٤٧.٨٪. تم تثبيت إنزيم الليبيز على حبيبات من الكيتوزان مغلفة بطبقة من أكسيد الحديد المغناطيسي بحجم النانو، مرتبطة تقاطعيا بواسطة الجلوتارالدهيد. تم دراسة عدد مرات الإستخدام و كذلك فترة التخزين للإنزيم المثبت ووجد أن الإنزيم قد إحتفظ ب ٨٠٪ من نشاطه بعد إستخدامه ١٥ مرة و كذلك إحتفظ ب٩٨٪ من نشاطه بعد تخزينه لمدة ٣٠ يوم على درجة حرارة ٤°C. تم إستخدام الإنزيم المثبت لتخليق إستر سكريات الجلوكوز أو الفراكتوز أو المانيتول مع حمض الأوليك و تم تحليل النتائج بإستخدام جهاز الكروماتوجرافي السائل- مطياف الكتلة. هذه الدراسة أوضحت أنه يمكن إستخدام إنزيم الليبيز المنتج بواسطة *Fusarium solani* في العديد من الصناعات التي تستخدم مذيبات عضوية أو درجات حموضة قلوية مثل صناعة المنظفات.