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IMMOBILIZATION AND CHARACTERIZATION OF A THERMOSTABLE β-GLUCOSIDASE FROM *ASPERGILLUS TERREUS* NRRL 265

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ABSTRACT

Partially purified β-glucosidase from *Aspergillus terreus* NRRL 265 was immobilized by entrapment in calcium-alginate beads. The activity of the free and immobilized enzymes as a function of pH, temperature, and periodic use were compared. Whey permeate, a by-product of cheese industry, was served as an inexpensive medium, which made the process economical and reduced the cost of enzyme production and also reduced the environmental pollution. The results indicated that, the immobilized β-glucosidase was retained about 73 % of the original activity exhibited by the free enzyme. The optimum temperature for the enzyme activity was improved by 5ºC after immobilization. Immobilized β-glucosidase was exhibited great thermal stability, whereas, at 70ºC, the free enzyme lost its activity after 30 min of incubation, while the immobilized enzyme showed more stability in comparison to the free form as it retained about 13.4 % of its initial activity under the same conditions. Moreover, the pH stability was improved following immobilization, whereas, the immobilized enzyme was stable in pH ranging from 4.0 to 7.0 with no change in activity, while its stability slightly decreases for more alkaline or acidic conditions (retaining 82.4 % and 67.4 % of the initial activity at pH 8.0 and 3.5, after 1 h of incubation). The results also indicated the possibility of reusing Ca alginate-immobilized β-glucosidase in industrial applications for 10 cycles with 53.7 % retained activity.

Keywords: *Aspergillus terreus* NRRL 265, β-glucosidase, immobilization, characterization

INTRODUCTION

Complete cellulose hydrolysis requires a synergistic action of exo-glucanases, endo-glucanases and β-glucosidase. β-glucosidase (β-D-glucoside glucohydrolase; E C 3.2.1.21), catalyzes the hydrolysis of β-1,4-glycosidic linkage in various disaccharides, oligosaccharides, alkyl- & aryl-β-D-glucosides **(Karagulyan *et al.*, 2008)**. This enzyme is widely distributed in all living kingdoms; it occurs ubiquitously in mammals, plants and microbial kingdoms including bacteria, yeasts, and fungi. However, microbial β-glucosidase attracts increasing attention owing to its potential application in the production of aromatic compounds, in the stabilization of juices and beverages, in the improvement of the organoleptic properties of food and feed products, in biomass degradation, in the production of fuel ethanol from cellulosic agricultural residues, and in the synthesis of alkyl- and aryl-glycosides from natural polysaccharides or their derivatives and alcohols, by reversed hydrolysis or trans-glycosylation, leading to products with applications in pharmaceutical, cosmetic, and detergent industries **(Gargouri *et al.*, 2004; Langston *et al.*, 2006; Nakkharat and Haltrich, 2006; Yeoman *et al.*, 2010; Saritha and Lata 2012)**. Microbial β-glucosidase can also be used to induce a loss of color of red fruit juices e.g. raspberry, strawberry, blackberry, cramberry and grapes. The decolorization is due to the breaking of linkage between the sugar residue and anthocyanidin which degrades spontaneously into colorless compounds (**Mazza and Minati, 1994; Chang *et al.*, 2008)**. In addition, β-glucosidase could be a useful tool for altering the form and properties of flavonoids (naturally occurring plant polyphenols) in order to positively affect their bioavailability and/or their biological activities in humans **(Crozier *et al.*, 1997; Price *et al.*, 1998; Mamma *et al.*, 2004)**. This enzyme is also vital for many biological processes, such as cyanide-based biodefense (CN releasing from cyano-glucoside) and degradation of various potentially harmful metabolites (e.g. glycosylceramides) **(Bhatia *et al.*, 2002)**. However, the high cost of enzyme production limits its use in the production of soluble sugars **(Zaldivar *et al.*, 2001)**. Hence, immobilization of enzyme in solid carrier offers the prospect of cost savings and widens the flexibility of process design, by enabling continuous operation (or multiple cycles of batch operation on a drain-and-fill basis) and simplifying downstream processing **(Sheldon, 2007; Fernandes, 2010)**. Immobilization of enzymes also enables improvements in thermo-stability of enzyme (thereby reducing enzyme inactivation), besides, the separation and recovery of enzyme is easy and convenient **(Bayramoglu *et al.*, 2003; Mazzuca *et al.*, 2006; El-Tanash *et al.*, 2011)**. Furthermore, the cost of β-glucosidase-based technology can also be reduced by increasing the enzyme reusability and its stability **(AbdEl-Ghaffar and Hashem, 2010)**. Thus, enzyme immobilization is one of the most promising techniques for highly efficient and economically competent biotechnological processes in the field of environmental monitoring, biotransformation, diagnostics, pharmaceutical and food industries **(Sheldon, 2007)**. Whey permeate, a by-product of the cheese industry, was served as an inexpensive medium. Whereas, a large amount of whey results from cheese production and the cost associated with disposing this large volume of whey is substantial. Furthermore, the high chemical oxygen demand (COD) [50 Kg O_2 / Ton] of whey permeate makes its disposal a pollution problem, leading to research into whey permeate utilization **(Liu *et al.*, 2005)**. Therefore, the objective of the present study was to immobilize β-glucosidase produced from *Aspergillus terreus* NRRL 265, grown on whey permeate, by entrapment in calcium alginate beads. Some kinetic parameters of the immobilized and free β-glucosidase were also investigated in order to explore the benefits of immobilization of enzymes.

MATERIAL AND METHODS

Microorganism

The fungal strain *Aspergillus terreus* NRRL 265, previously reported as a hyper-β-glucosidase producing fungal strain **(Elshafei *et al.*, 2011)**, was obtained from Northern Regional Research Laboratory (NRRL), Peoria, Illinois, USA. The culture was grown for 7 days on potato dextrose agar slants (PDA) at 28°C, and then maintained at 4ºC. The slants were subcultured routinely every 3-4 weeks interval.

Whey permeate

Whey permeate, a by-product of the cheese production, was obtained from Egyptian Dairy Industry El-Amiria, Cairo, Egypt. The average composition of whey permeate is as follows: Lactose (4 - 5 g %), ash (0.7 g %), with an initial pH value of 6.5.

Chemicals and buffers

p-Nitrophenyl-β-D-glucopyranoside (*p*-NPG) and sodium alginate were purchased from Sigma Chemicals Company (St. Louis, MO, U.S.A.). Buffers were prepared according to the method presented by **Gomori (1955)**. All other chemicals were of analytical grade and of high purity.

Culture conditions

Aspergillus terreus NRRL 265 was grown on PDA agar slants for 7 days at 28°C. Conidia were scraped and 5.0 ml of sterile distilled water was added to each slant. One ml aliquot (v/v) of inoculum size ($1x10^6$ spores/ml) was used to inoculate 50 ml of whey permeate supplemented with 0.075% NH_4Cl and 0.05% yeast extract in 250 ml Erlenmeyer flasks. The initial pH was adjusted at pH 6.0 before autoclaving (121°C for 20 min). The inoculated flasks were incubated for 4 days at 30ºC in a static condition.

Preparation of cell-free extracts

The mycelial biomass were harvested by filtration, rinsed thoroughly with distilled water, and blotted dry with absorbent paper then ground with approximately twice its weight of cold washed sand in a chilled mortar and extracted with cold sodium citrate buffer (0.05 M, pH 5.0) according to the method presented by **Sebald *et al*. (1979)**. Thereafter, the slurry obtained was centrifuged at 5500 rpm for 15 min at 4ºC. The supernatant was used as the crude intracellular enzyme preparation.

Partial purification of β-glucosidase

The cell free extract obtained from the previous step was precipitated with cold absolute ethanol (chilled to -20ºC) 50 % (v/v). The precipitation was carried out at 4ºC under constant stirring for 30 min. The precipitated protein was dialyzed against sodium citrate buffer (0.05 M, pH 5.0) for 24 h at 4°C by using a dialysis bag, size 3, Inf Dia. 20/32-15.9 mm, Medicell International Ltd, the buffer was changed three times during dialysis process.

β- Glucosidase assay

β-Glucosidase activity was determined photometrically using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) as a substrate. The *p*-nitrophenol (*p*-NP) produced from the degradation of *p*-NPG by enzyme activity was measured according to the method of **Bergham and Pettersson (1974)**. The enzyme units were calculated from standard curve using different concentrations of *p*-NP. One unit of enzyme activity expressed as International Unit/ml (U/ml) was defined as the amount of enzyme liberating one micromole (µmole) *p*-NP/min. under the standard assay conditions **(Mamma *et al*., 2004)**.

Protein determination

Protein concentration was determined according to **Bradford (1976)**.

Immobilization method

Entrapment in Ca-alginate beads

An equal volume of the dialyzed enzyme (5 ml) and 3 % sodium alginate solution was mixed thoroughly. The entrapment was carried out by dripping the enzyme-alginate mixture through a Pasteur pipette (1 mm diameter) from a height of 20 cm drop wise into a gently stirred 50 ml of $CaCl_2$ solution (0.2 M) with continuous shaking. The formed beads were retained in stirred $CaCl_2$ solution for 2 h then washed 3-4 times with distilled water and finally with 0.05 M sodium citrate buffer pH 5.0 as reported by **Dey *et al*. (2003)**. The beads were dried and weighted for further studies.

Immobilization calculation

The efficiency of an immobilization process can be measured by the criteria that high percentage of the enzyme must be initially retained in the gel matrix **(Singh *et al*., 2011)**. The enzyme activity was recorded as the U/g bead.
Initial activity of the free enzyme = 16.74 U/ml.
Volume of enzyme solution taken for immobilization = 5 ml.
Weight of beads formed after immobilization of enzyme solution = 10.7 g.
Enzyme solution entrapped in 2 g beads = 0.935 ml.
Therefore, enzyme entrapped = 15.644 U/2 g beads (on the basis of free enzyme).
Activity of immobilized enzyme obtained in 2g beads = 11.428 Units/ 2 g beads.
Therefore, enzyme activity after entrapment (Immobilization efficiency) = 73 %.

Effect of sodium alginate concentration

Various concentrations of sodium alginate were used to acquire beads with greater stability. Sodium alginate solutions (1 - 4 %) were prepared. The rest of the procedure for enzyme immobilization was made as that stated previously.

Optimal reaction time

This experiment was carried out to identify the optimal incubation time on the activity of free and immobilized β-glucosidase by incubating the standard reaction mixtures in a period of time ranging from 5 to 40 min.

Temperature profile

Both free and immobilized β-glucosidase were incubated separately in 1 ml of 5 mM *p*-NPG dissolved in sodium citrate buffer (0.05 M, pH 4.8) at different temperatures ranging from 30 - 80°C for 10 min.

Thermal stability

Thermostability studies were carried out by pre-incubating the free and immobilized β-glucosidase at different temperatures (50, 60 and 70°C) for different time intervals (5 - 60 min), and the residual activities were determined under the standard assay conditions. The non-heated enzyme was considered as a control (100 %).

pH profile

The optimum pH for free and immobilized enzymes was determined by measuring the enzyme activity at different pH values ranging from pH 3.0 to 8.0 using suitable buffers, namely sodium citrate buffer (0.05 M, 3.0 - 7.0) and sodium phosphate buffer (0.05 M, 7.0 - 8.0), with 5 mM *p*-NPG as a substrate under the standard assay conditions.

pH stability

The pH stability was examined after pre-incubating both enzymes for 2 h at 4ºC in sodium-citrate buffer (0.05 M, pH 3.0 - 6.0), sodium-phosphate buffer (0.05 M, pH 6.0 - 7.0) and phosphate buffer (0.05 M, pH 7.0 - 8.0). Afterwards, pH was adjusted to the value of the standard assay system (pH 5.0), and the residual activities were assayed under the standard conditions for free and immobilized β-glucosidase.

Repeated batch hydrolysis

Consecutive batch runs were performed under the optimum assay conditions using *p*-NPG (5 mM) in sodium-citrate buffer (0.05 M, pH 5.0). After each cycle, the immobilized biocatalyst was separated and washed with the same buffer. The reaction medium was then replaced with a fresh medium. The activity of the freshly prepared immobilized enzyme in the first run was defined as 100%.

Statistical analysis

Statistical analysis was carried out according to the method described by **Kenney and Keeping (1962)**, and the data were expressed as the mean $\pm$ S. D of three replicates.

RESULTS AND DISCUSSION

Effect of sodium alginate concentration on immobilization efficiency

Calcium alginate gel was chosen as a carrier for the enzyme entrapment due to its non-toxicity, high mechanical stability, high porosity for substrate and product diffusion and above all the simple procedural requirements for immobilization. It has been reported that the degree of cross linking of the gelling agent affects the pore size of the beads **(Longo *et al*., 1992)**. Therefore, various concentrations of sodium alginate (1 – 5 %) were used to acquire beads with greater stability and highest immobilization efficiency. The immobilization efficiency was found to be highest for 3 % sodium alginate solution (**Fig. 1**). However, at lesser concentration (1 - 2 %), the beads were unstable and not formed properly. Besides, maximum leakage of the enzyme occurred, owing to the large pore size of fragile calcium alginate beads. On the other hand, at higher concentration of sodium alginate (4 - 5 %), the enzyme activity was comparatively low, which might be due to the high viscosity of the enzyme entrapped beads, which decreased the pore size and thus hindered the penetration of the substrate into the beads. In this concern, **Dey *et al*. (2003)** investigated that, the increase in sodium alginate concentration interfered the entry of the substrate into the beads; that led to lower immobilization efficiency. In addition it is worth to mention that, it was difficult to prepare immobilized beads with higher concentration of sodium alginate.

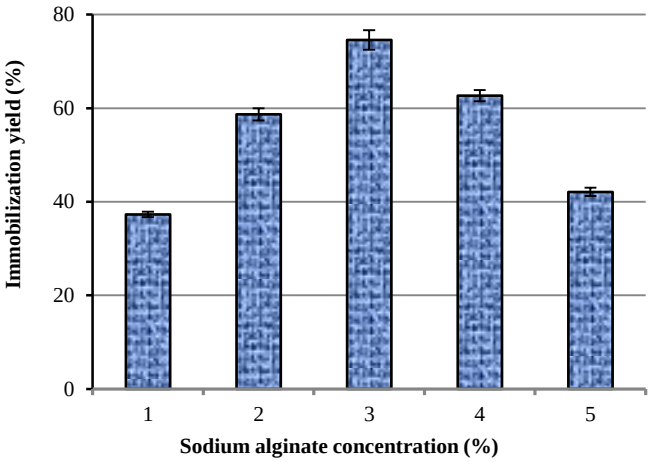

Figure 1 Effect of Sodium-alginate concentration on immobilization efficiency

Effect of reaction time

The optimum activity of both enzymes was achieved after 30 min. (**Fig. 2**), this result suggesting that the reaction rate of enzyme and substrate is independent to immobilization, as the diffusion limitation for substrate has not been found after the entrapment of enzyme in calcium alginate gel. It may conclude that pore size of the beads is optimum for the passage of substrate into the beads **(Riaz *et al.*, 2009)**.

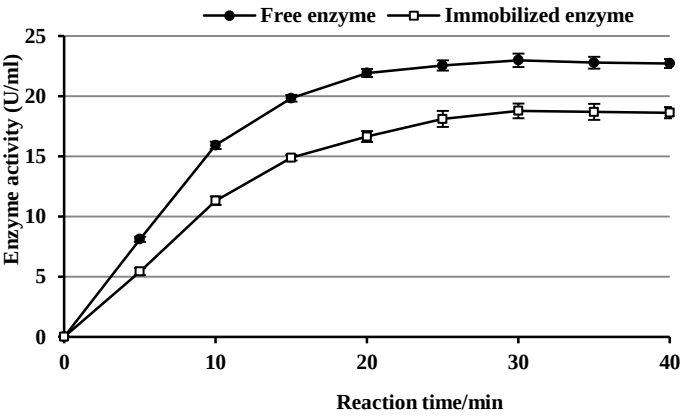

Figure 2 Effect of reaction time on the activity of free and immobilized β-glucosidase

Temperature activity profile

The temperature dependence of the free and immobilized β-glucosidase was studied in the temperature range of 30 to 80ºC. **Fig. 3** clearly illustrated that, the operating temperature of immobilized enzyme was raised from 60 to 65ºC. The higher temperature profile of the immobilized enzyme might result from a lower temperature in the gel microenvironment compared to the bulk solution. The increase in optimum temperature might be due to the change in the physical and chemical properties of the enzyme **(Peng *et al.*, 2005; AbdEl-Ghaffar and Hashem, 2010)**. The free enzyme lost about 75 % from its activity at 80ºC, while at the same temperature; the immobilized enzyme activity retained 47 %. This result indicates the effectiveness of the carriers in protecting the enzyme activity under higher temperature conditions. In congruent with this result, **AbdEl-Ghaffar and Hashem (2010)** reported that, the free cellulase exhibited an optimum temperature of 50ºC that shifted to 60ºC after immobilization on chitosan-L-glutamic acid-GDA (1 %).

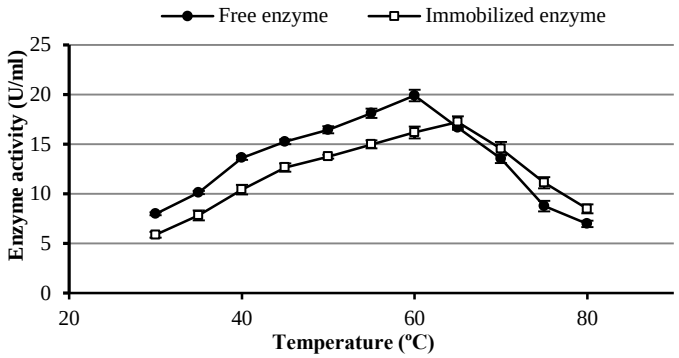

Figure 3 Effect of temperature on the activity of free and immobilized β-glucosidase

pH activity profile

The optimal pH for immobilized and free enzymes was 5.0 (**Fig. 4**). However, compared to the free enzyme, the immobilized enzyme showed changes in pH profiles as it is less sensitive to modification of the reaction conditions than the free enzyme. Such effects are usually investigated when the enzyme is covalently bound to a support or entrapped in a porous matrix. Similar patterns were reported earlier by **Tu *et al.* (2006)** and **Figueira *et al.* (2011)**, whereas, they found that, the optimum pH of β-glucosidase activity is not altered with immobilization. In general, difference in pH profiles of the immobilized enzymes depends on the applied immobilization methods and on the carriers used for the enzyme immobilization **(Abdel-Naby, 1999)**. On the other hand, **Riaz *et al.* (2009)** reported that, optimum pH for entrapped α-amylase activity was shifted from 7.0 to 7.5 after immobilization.

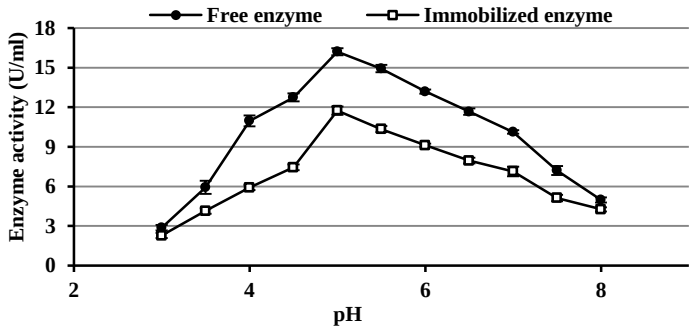

Figure 4 Effect of pH on the activity of free and immobilized β-glucosidase

Thermal stability of the free and immobilized β-glucosidase

The kinetic stability of both enzymes was compared and the results were tabulated in **Table 1**, from which it was clearly indicated that, the activity of the immobilized and free enzymes retained 100 % of their activities after 1 h of incubation, in absence of substrate, at 50ºC. While, at 60ºC, the immobilized and free β-glucosidase activities were retained 85.4 and 78.2 %, respectively. Furthermore, at 70ºC, the free enzyme lost its activity after 30 min of incubation, while the immobilized enzyme showed more stability in comparison to the free form as it retained about 13.4 % of its initial activity under the same conditions. These results demonstrated that, the immobilized form was inactivated at a slower rate than the native form, whereas, the thermal stability of immobilized β-glucosidase increased considerably as a result of immobilization in alginate gel. Enzyme stabilization by immobilization may also be caused by the existence of a local environment for the immobilized enzymes which is less damaging than the bulk solution condition **(Figueira *et al.*, 2011)**. A significant improvement in the stability of β-glucosidase was noted upon immobilization as reported by **Itoh *et al.* (2010)** and **Singh *et al.* (2011)**. The immobilization support can have a protecting effect from heat when enzyme inactivation occurs **(Chang *et al.*, 2008)**. **Figueira *et al.* (2011)** reported that, the immobilization causes an increase in enzyme rigidity, which is reflected by an increase in stability towards denaturation upon raising the temperature.

Table 1 Thermal stability of free and immobilized β-glucosidase enzyme

Temp. (ºC)	Relative activity (%)											
	Free β-glucosidase						Immobilized β-glucosidase					
	5 min	10 min	15 min	30 min	45 min	60 min	5 min	10 min	15 min	30 min	45 min	60 min
50	100	100	100	100	100	100	100	100	100	100	100	100
60	100	100	100	91.7	85.6	78.2	100	100	100	95.6	90.2	85
70	43.2	25.6	15.8	0.0	0.0	0.0	55.7	36.2	26.7	13.4	0.0	0.0

pH stability

The pH stability was improved following immobilization, whereas, the immobilized enzyme was stable in pH ranging from 4.0 to 6.5 with no change in activity, while its stability slightly decreases for more alkaline or acidic conditions (retaining 82.4 % and 42.1 % of the initial activity at pH 8.0 and 3.0, after 1 h of incubation) (**Table 2**). On the other hand, the free enzyme was less stable regarding pH as it retained about 72.6 % and 37.8 % of the its initial activity at pH 8.0 and 3.0, under the same conditions. In this concern, **Chellapandian (1998)** reported the same observation for immobilized alkaline protease on vermiculite, which exhibited a higher stability under acidic and alkaline conditions, compared to the free enzyme.

Table 2 pH stability of free and immobilized β-glucosidase enzyme

pH	Relative activity (%)	
	Free β-glucosidase	Immobilized β-glucosidase
3.0	37.8	42.1
3.5	61.4	67.4
4.0	88.7	100
4.5	100	100
5.0	100	100
5.5	100	100
6.0	91.8	100
6.5	86.6	100
7.0	84.5	100
7.5	81.2	87.5
8.0	72.6	82.4

Repeated hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside by immobilized enzyme

Immobilized β-glucosidase was used repeatedly to hydrolyze *p*-NPG, and reusability examined because of its importance for repeated applications in a batch or a continuous operation. **Table 3** illustrates the effect of repeated use on the activity of β-glucosidase, from which it was observed that, the immobilized enzyme retained about 54 % of its original activity after 10 cycles of reuse (every run 10 min). This result is in congruent with that obtained by **AbdEl-Ghaffar and Hashem (2010)** for cellulase immobilization on chitosan-GDA (1%), which retained about 60 % of its initial activity. Furthermore, **Dinçer and Telefoncu (2007)** reported that, the immobilized cellulase on modified polyvinyl alcohol coated chitosan beads retained 52 % of its initial activity after 8 cycles of reuse. Similarly, **Ahmed *et al.* (2013)** reported that, the sponge-immobilized β-glucosidase, produced by *Aspergillus niger*, was repeatedly used to hydrolyze cellobiose, and the enzyme retained about 67.32 and 51.04 % of its initial activity after 5 and 8 cycles, respectively.

Table 3 Repeated hydrolysis of *p*-NPG by immobilized β-glucosidase

No. of cycle	Relative activity (%)
1	100
2	97.3
3	91.8
4	88.2
5	79.6
6	72.2
7	68.5
8	62.4
9	58.8
10	53.7
11	46.1

Mechanical stability

Seven to eight beads of calcium alginate were centrifuged at 1200 rpm for 10 min. All the beads remained intact and none were broke or adhered.

CONCLUSION

A filamentous fungal strain *Aspergillus terreus* NRRL 265 is capable of producing a thermostable β-glucosidase which can be used in several biotechnological processes. This study indicates that, 3 % sodium alginate was the most suitable concentration for immobilization of β-glucosidase. The immobilized enzyme has a higher optimum temperature as compared to the free enzyme. In addition, the immobilization process enhanced the thermal properties of the enzyme, besides the possibility of reusing Ca alginate-immobilized β-glucosidase in industrial applications for 10 cycles with 53.7 % retained activity. Therefore, we can conclude that, the enzyme immobilization is one of the most promising techniques for highly efficient and economically competent biotechnological processes in pharmaceutical and food industries.

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REFERENCES

ABD EL-GHAFFAR, M. A., HASHEM, M. S. 2010. Chitosan and its amino acids condensation adducts as reactive natural polymer supports for cellulase immobilization. *Carbohydr Polymers*, 81, 507-516. http://dx.doi.org/10.1016/j.carbpol.2010.02.025

ABDEL-NABY, M. A. 1999. Stabilization of cellobiase by covalent coupling to soluble polysaccharide. *Microbiol Res*, 154, 213-218. http://dx.doi.org/10.1016/s0944-5013(99)80017-2

AHMED, S. A., EL-SHAYEB, N. M. A. HASHEM, A. M., SALEH, S. A., ABDEL-FATTAH, A. F. 2013. Biochemical studies on immobilized fungal β-glucosidase. *Braz J Chem Eng*, 30, 747-758. http://dx.doi.org/10.1590/s0104-66322013000400007

BAYRAMOGLU, G., AKGOL, S., BULUT, A., DENIZLI, A., ARICA, M. 2003. Covalent immobilization of invertase onto a reactive film composed of 2-hydroxy ethyl methacrylate and glycidyl methacrylate: Properties and application in a continuous flow system. *Biochem Eng J*, 14, 117-126. http://dx.doi.org/10.1016/s1369-703x(02)00170-5

BERGHAM, L. E. R., PETTERSSON, L. G. 1974. The mechanism of enzymatic degradation, isolation and some properties of a β-glucosidase of *Trichoderma koningii*. *Eur J Biochem*, 46, 295-305. http://dx.doi.org/10.1111/j.1432-1033.1974.tb03621

BHATIA, Y., MISHRA, S., BISARIA, V. S. 2002. Microbial β-glucosidase: Cloning, properties, and applications. *Crit Rev Biotechnol*, 22, 375-407. http://dx.doi.org/10.1080/07388550290789568

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72,248-254. http://dx.doi.org/10.1016/0003-2697(76)90527-3

CHANG, M. Y., KAO, C. H., JUANG, R. S. 2008. Thermal inactivation and reactivation of β-glucosidase immobilized on chitosan-clay composite. *Int J Biol Macromol*, 43, 48-53. http://dx.doi.org/10.1016/j.ijbiomac.2007.10.004

CHELLAPANDIAN, M. 1998. Preparation and characterization of alkaline protease immobilized on vermiculite. *Process Biochem*, 33, 169-173. http://dx.doi.org/10.1016/s0032-9592(97)00043-5

CROZIER, A., LEAN, M. E. J., MCDONALD, M. S., BLACK, C. 1997. Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuce and celery. *J Agricult Food Chem*, 45, 590-595. http://dx.doi.org/10.1021/jf960339y

DEY, G., SINGH, B., BANERJEE, R. 2003. Immobilization of α-amylase produced by *Bacillus circulans* GRS313. *Braz Arch Biol Technol*, 46, 167-176. http://dx.doi.org/10.1590/s1516-89132003000200005

DINÇER, A. TELEFONCU, A. 2007. Improving the stability of cellulase by immobilization on modified polyvinyl alcohol coated chitosan beads. *J Mol Catal B: Enzymatic*, 45, 10-14. http://dx.doi.org/10.1016/j.molcatb.2006.10.005

ELSHAFEI, A. M., HASSAN, M. M., MORSI, N. M., EL-GHONEMY, D. H. 2011. Purification and some kinetic properties of β-glucosidase from *Aspergillus terreus* NRRL 265. *Afr J Biotechnol*, 10, 19556-19569. http://dx.doi.org/10.5897/ajb10.2617

EL-TANASH, A. B., SHERIEF, A. A., NOUR, A. 2011. Catalytic properties of immobilized tannase produced from *Aspergillus acuteatus* compared with free enzyme. *Braz J Chem Eng*, 28, 381-391. http://dx.doi.org/10.1590/s0104-66322011000300004

FERNANDES, P. 2010. Enzymes in food processing: a condensed overview on strategies for better biocatalysts. *Enzyme Res*, 20, 1-19. http://dx.doi.org/10.4061/2010/862537

FIGUEIRA, J. A., DIAS, F. F. G., SATO, H. H., FERNANDES, P. 2011. Screening of supports for the immobilization of β-glucosidase. *Enzyme Res Article* ID 642460, 1-8. http://dx.doi.org/10.4061/2011/642460

GARGOURI, M., SMAALI, I., HAUGARD, T., LEGOY, M. D., MARZOAK, N. 2004. Fungus β-glucosidases: Immobilization and use in alkyl-β-glycoside synthesis. *J Mol Catal B: Enzymatic*, 29, 89-94. http://dx.doi.org/10.1016/j.molcatb.2003.11.020

GOMORI, G. 1955. Preparations of buffers for the use in enzyme studies. *Methods Enzymol*, 1, 138-146. http://dx.doi.org/10.1016/0076-6879(55)01020-3

ITOH, T., ISHII, R., MATSUURA, S., MIZUGUCHI, J., HAMAKAWA, S., HANAOKA, T. A., TSUNODA, T., MIZUKAMI, F. 2010. Enhancement in thermal stability and resistance to denaturants of lipase encapsulated in mesoporous silica with alkyltrimethyl ammonium (CTAB). *Colloids Surfaces B*, 75, 478-482. http://dx.doi.org/10.1016/j.colsurfb.2009.09.022

KARAGULYAN, H. K., GASPARYAN, V. K., DECKER, S. R. 2008. Immobilization of fungal β-glucosidase on Silica gel and Kaolin carriers. *Appl Biochem Biotechnol*, 146, 39-47. http://dx.doi.org/10.1007/s12010-007-8065-3
KENNEY, J. F., KEEPING, E. S. 1962. *"The Standard Deviation" and "Calculation of the Standard Deviation" §6.5 - 6.6 in Mathematics of Statistics, Pt. 1*, 3rd ed. Princeton, NJ: Van Nostrand, 77-80. http://dx.doi.org/10.1002/0471743984.vse6698
LANGSTON, J., SHEEHY, N., XU, F. 2006. Substrate specificity of *Aspergillus oryzae* family 3 β- glucosidase. *Biochim Biophys Acta*, 1764, 972-978. http://dx.doi.org/10.1016/j.bbapap.2006.03.009
LIU, J., YUAN, X., ZENG, G., SHI, J., CHEN, S. 2005. Effect of biosurfactant on cellulase and xylanase production by *Trichoderma viride* in solid substrate fermentation. *Process Biochem*, 41, 2347-2351. http://dx.doi.org/10.1016/j.procbio.2006.05.014
LONGO, M. A., NOVELLA, I. S., GARCIA, L. A., DIAZ, M. 1992. Diffusion of proteases in calcium alginate beads. *Enzyme Microb Technol*, 14, 586-590. http://dx.doi.org/10.1016/0141-0229(92)90131-7
MAMMA, D., HATZINIKOLAOU, D. G., CHRISTAKOPOULOS, P. 2004. Biochemical and catalytic properties of two intracellular β-glucosidases from the fungus *Penicillium decumbens* active on flavonoid glucosides. *J Mol Catal B: Enzymatic*, 27, 183-190. http://dx.doi.org/10.1016/j.molcatb.2003.11.011
MAZZA, G., MINIATI, E. 1994. *Small fruits. In: Anthocyanins in Fruits*. Vegetables and Grains (Mazza, G. and Miniati, E., Eds.). CRC. Press, London. 83-190. http://dx.doi.org/10.1002/food.19940380317
MAZZUCA, S., GIORNO, L., SPADAFORA, A., MAZZEI, R., DRIOLI, E. 2006. Immunolocalization of β-glucosidase immobilized within polysulphone capillary membrane and evaluation of its activity in situ. *J Membr Sci*, 285, 152-158. http://dx.doi.org/10.1016/j.memsci.2006.08.018
NAKKHARAT, P., HALTRICH, D. 2006. Purification and characterization of an intracellular enzyme with β-glucosidase and β-galactosidase activity from the thermophilic fungus *Talaromyces thermophilus* CBS 236.58. *J Biotechnol*, 123, 304-313. http://dx.doi.org/10.1016/j.jbiotec.2005.12.015
PENG, Y., ZHI-KANG, X., ALI-FU, C., JIAN, W., PATRICK, S. 2005. Chitosan-tethered poly (acrylonitritleco-maleic acid) hollow fiber membrane for lipase immobilization. *Biomaterials*, 26, 6394-6403. http://dx.doi.org/10.1016/j.biomaterials.2005.04.019
PRICE, K. P., CASUSCELLI, F., COLQUHOUN, I. J., RHODES, M. J. 1998. Composition and content of flavonol glycosides in *Brassica olearacea* and their fate during cooking. *J Sci Food Agricult*, 77, 468-472. http://dx.doi.org/10.1002/(sici)1097-0010(199808)77:4%3C468::aid-jsfa66%3E3.3.co;2-2
RIAZ, A., QADER, S. A., ANWAR, A., IQBAL, S. 2009. Immobilization of a Thermostable á-amylase on Calcium Alginate Beads from *Bacillus Subtilis* KIBGE-HAR. *Aust J Basic Appl Sci*, 3, 2883-2887. http://dx.doi.org/10.5580/11a0
SARITHA, M., LATA, A. A. 2012. Biological pretreatment of lignocellulosic substrates for enhanced delignification and enzymatic digestibility. *Indian J Microbiol*, 52, 122-130. http://dx.doi.org/10.1007/s12088-011-0199
SEBALD, W., NEUPERT, W., WEISS, H. 1979. Preparation of *Neurospora crassa* Mitochondria. *Meth Enzymol*, 55, 144-148. http://dx.doi.org/10.1016/0076-6879(79)55020-4
SHELDON, R. A. 2007. Enzyme immobilization: the quest for optimum performance. *Adv Synthesis Catal*, 349, 1289-1307. http://dx.doi.org/10.1002/adsc.200700082
SINGH, R. K., ZHANG, Y. W., NGUYEN, N. P. T., JEYA, M., LEE, J. K. 2011. Covalent immobilization of β-1,4-glucosidase from *Agaricus arvensis* onto functionalized silicon oxide nanoparticles. *Appl Microbiol Biotechnol*, 89, 337-344. http://dx.doi.org/10.1007/s00253-010-2768
TU, M., ZHANGE, X., KURABI, A., GILKES, N., MABEE, W. W., SADDLER, J. 2006. Immobilization of β-glucosidase on Eupergit C for lignocellulose hydrolysis. *Biotechnol Lett*, 28, 151-156. http://dx.doi.org/10.1007/s10529-005-5328-3
YEOMAN, C. J., HAN, Y., DODD, D., SCHROEDER, C. M., MACKIE, R. I., CANN, K. I. 2010. Thermostable enzymes as biocatalysts in the biofeul industry. *Adv Appl Microbiol*, 70, 1-55. http://dx.doi.org/10.1016/s0065-2164(10)70001-0
ZALDIVAR, M., VELÁSQUEZ, J. C., CONTRERAS, I., PÉREZ, L. M. 2001. *Trichoderma aureoviride* 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and/or biocontrol. *J Biotechnol*, 4, 160-168. http://dx.doi.org/10.2225/vol4-issue3-fulltext-7