CHEMICAL ENGINEERING TRANSACTIONS
VOL. 38, 2014
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Taj Keshavarz

ISBN 978-88-95608-29-7; **ISSN** 2283-9216
DOI: 10.3303/CET1438069

Immobilization of Endo (1→4) β-D-Glucanase from *Bacillus Licheniformis* KIBGE-IB2 Using Agar-Agar as Support for Continuous Use

Asad Karim, Shah Ali Ul Qader, Asif Nazwaz, Afsheen Aman

The Karachi Institute of Biotechnology and Genetic Engineering , (KIBGE), University of Karachi, Karachi-75270, Pakistan

Endo (1→4) β-D-glucanase [EC 3.2.14] is a type of cellulase, which randomly cleaves the β (1→4) glycosidic linkages in cellulose polymer chain. It is widely used in different industries such as, biofuel, food, textile, paper and pulp industries. There are several limitations in using soluble enzymes in industrial processes, for instance, they show low stability under harsh operational conditions and the enzyme recovery becomes difficult for continuous use. Immobilization technology is one of the solutions that not only overcome the aforementioned problems, but also makes the process more cost effective. Therefore, the current study was designed to study the effect of immobilization using the entrapment technique for endo (1→4) β-D-glucanase from *B. licheniformis* KIBGE-IB2. For this purpose, nontoxic, non-protein reactive bio-polymer agar-agar was used. A maximum immobilization yield of 66.0% was achieved at 2.0 g % (w/v) agar-agar. The immobilized enzyme exhibited broader pH and temperature activity profile as compared to soluble enzyme. The temperature optimum of the soluble enzyme was found to be 60°C and it shifted to 70°C after entrapment. Optimal pH for both the systems remains unchanged (pH-6.0). The immobilized enzyme shows greater thermal stability in the range of 50°C to 80°C, with reference to soluble enzyme. The entrapped enzyme in agar-agar matrix retained its activity for 8 successive cycles. The results indicate a possibility of employing matrix entrapped endo (1→4) β-D-glucanase from *B. licheniformis* KIBGE-IB2 for various industrial applications.

1. Introduction

Cellulose is a major complex structural material present in the plant cell wall and it is considered to be the most abundant renewable bio-polymer source on earth. In nature, there are certain types of organism, including bacterial species which break down cellulosic material to soluble sugar by producing an enzyme known as cellulase. Cellulases are a group of enzymes (Endo-1, 4-β-D-glucanase [EC 3.2.1.4], Exo-1, 4-β-D-glucanase [EC 3.2.1.91] and β-glycosidase [EC 3.2.1.21]) that cleaves β (1→4) glycosidic linkages in cellulose polymer. The endo-1, 4-β-D-glucanase hydrolyzes the β (1→4) glycosidic linkages, producing reducing and non-reducing ends. The exo-1, 4-β-D-glucanase utilizes the reducing and non-reducing ends to produce cellobiose units. Finally, β-glycosidase cleaves cellobiose to two glucose molecules.
The market demand for large scale production of cellulase is increasing for the enzymatic hydrolysis of cellulosic waste material to important bio-products. Cellulase is used in food industries for extraction of beer, fruit juice, vegetable juice, seed oil and also helps in improving the nutritive quality of bakery products (Beilen and Li, 2002; Bhat, 2000). Moreover, it is also used in other industries including; textile industry (Gusakov *et* al., 2000), pulp and paper industry (Hildén *et al*., 2005).
Most of the industrial processes require high temperature (above 60ºC) for enzymatic reactions and the majority of cellulase cannot be used for industrial purpose due to the high temperature sensitivity (Karnchanatat *et al*., 2008). Thus, cellulase having higher stability in a broad range of pH and temperature is beneficial for various industrial processes. The challenging problems beside enzyme stability are the cost of enzyme production and final product contamination due to the presence of soluble enzyme in the

Please cite this article as: Karim A., Qader S.A.U., Nawaz A., Aman A., 2014, Immobilization of endo (1→4) β-d-glucanase from bacillus licheniformis kibge-ib2 using agar-agar as support for continuous use, Chemical Engineering Transactions, 38, 409-414
DOI: 10.3303/CET1438069

reaction medium. The separation of the enzyme from product is a very difficult and costly process. Therefore, enzyme immobilization technique can be used to overcome aforementioned problems in different industries.

Endo-1, 4-β-D-glucanase has been immobilized by different physical and chemical methods such as cross-linking of the enzyme molecule (Barstow *et al*., 1997), co-polymerization (Yuan *et al*., 1999), fibre ultra-filtration (Ohlson *et al.*, 1984), aqueous two phase systems (Tjerneld *et al.,* 1991) and use of an enzyme carrier, such as non-porous ultrafine silica particles (Afsahi *et al.*, 2007).

In order to achieve maximum advantage of immobilization techniques, the selection of appropriate method is very important which ultimately causes no effect on enzyme basic activity and make more active sites available for enzyme substrate reaction (Mateo *et al*., 2007).

Entrapment method is a single step immobilization technique in which enzyme is physically confined within a matrix without altering its structural configuration. Hence, enzyme has less chance to loss its activity and stability in entrapment method. According to Mateo *et al*., (2007) simple protocols for enzyme immobilization and highly stabilized enzyme could be useful for enzymatic process.

In the current study, nontoxic, non-protein reactive and commonly available bio-polymer knows as agar-agar (agar) has been used for the entrapment of endo-1, 4-β-D-glucanase from *Bacillus licheniformis* KIBGE IB2.

2. Experimental

2.1 Microorganism

Bacillus licheniformis KIBGE-IB2 was collected from the culture bank of Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi.

2.2 Enzyme production

The bacterial strain was cultured in CMC (Carboxymethyl cellulose) containing medium: CMC, 0.5 % (w/v), Yeast extract, 1.5 % (w/v), Peptone, 1.5 % (w/v), K_2HPO_4, 0.5 % (w/v), $MgSO_4$, 1.0 % (w/v), $CaCl_2$, 0.0001 % (w/v), $Na_2HPO_4.2H_2O$, 0.5 % (w/v), $FeSO_4$, 0.001 % (w/v) and having initial pH-6.0. After incubation at 37°C for 48 hours, the bacterial cell mass was separated out by centrifugation at 40,248 × *g* at 4°C for 20 minutes.

2.3 Crude enzyme precipitation

Cell free filtrate (CFF) was precipitated using 50% saturation of ammonium sulphate at 4ºC. The precipitate was dissolved in 0.06M citrate buffer pH 6.0 and dialysed against the same buffer for overnight.

2.4 Immobilization of endo-1, 4-β-D-glucanase

Different concentrations of agar-agar (1.0 to 6.0%) were prepared in 0.06M citrate buffer pH 6.0 by heated in boiling water bath. After cooling between 40-45°C, 9.0 ml of partially purified endo-1, 4-β-D-glucanase was mixed with 9.0 ml agar-agar solution and immediately poured into a petri dish. After solidification at room temperature, the gel was cut into tablets of 5.0 (5.0 mm size and washed several times with buffer before use. The agar-agar tablets were stored at 4.0 ºC for further use.

2.5 Enzyme assay

The assay reaction mixture contains 0.25 g agar tablets containing enzyme and 1.0 ml substrate (0.5% CMC prepared in 0.06 M citrate buffer, pH-6.0) and mixed for 10 minutes at 60ºC. The amount of reducing sugar liberate during the reaction was determined using the DNS method and the absorbance was measured at 546 nm (Miller, 1959).

One unit of endo-1, 4-β-D-glucanase is defined as “the amount of enzyme required to liberate 1.0 µmol of glucose under standard assay conditions”.

2.6 Effect of temperature and pH

The effect of temperature and pH on the activities of soluble and immobilized endo-1, 4-β-D-glucanase were assayed by varying temperatures ranging from 30°C to 80°C and pH ranging from 5.0 to 10.0 pH.

2.7 Thermal stability

The thermal stability of soluble and immobilized endo-1, 4-β-D-glucanase were monitored at different temperatures (30°C to 80°C) for 120 minutes.

2.8 Kinetic parameters

K_m and V_{max} value was determined by varying the substrate concentration at constant pH and temperature using Lineweaver-Burk plot.

2.9 Reusability of immobilized enzyme

The reusability of immobilized endo-1, 4-β-D-glucanase was studied at 50°C using repeated batch process. After each cycle the agar-agar tablets were washed with buffer and were used for the next cycle. The immobilized enzyme activity of 1st cycle was defined as the control and attributed a residual activity of 100%.

2.10 Scanning electron microscopy

The surface morphology of agar-agar tablets before and after entrapment of the enzyme was observed by scanning electron microscope (SEM) images taken at different magnification. The micrographs were taken using SEM(JSM 6380A Jeol, Japan).

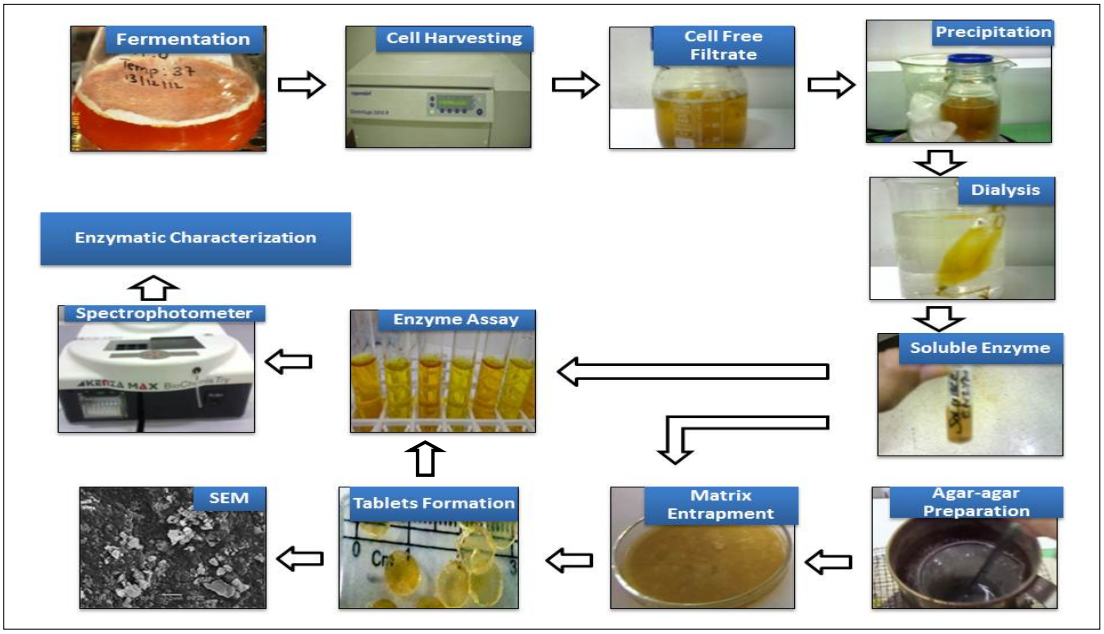

Figure 1: The complete schematic presentation of the current work

3. Results and Discussion

3.1 Effect of agar-agar concentration on immobilization

Different concentrations of agar-agar (0.5-3.0 g % w/v) were used for the acquisition of agar-agar tablets with greater stability and higher immobilization yield (Figure 2). The optimum immobilization yield (66.0%) was obtained at 2.0 g % agar-agar. The decline in immobilization yield above optimal concentration might be due to the decrease in the porosity of the agar-agar gel and caused diffusion limitation of the substrate. As it is evident from Figure 2 there was only about 10.6% immobilization yield at 0.5 g % (w/v) agar-agar and the tablets were very fragile and susceptible to damage during handling. The results imply that below 2.0 g % agar-agar the pore size might be increased in such a way that the enzyme leached out during washing step and ultimately resulted in decreased immobilization yield.

3.2 Temperature and pH profile

Temperature dependent activities of soluble and immobilized endo-1, 4-β-D-glucanase were studied in the range of 40°C to 80°C (Figure 3A). It was found that the relative activity of both soluble and immobilized enzymes increased, as the temperature was raised. The optimal temperature of entrapped endo-1, 4-β-D-glucanase was found to be 70ºC, which was slightly higher (10ºC) than soluble enzyme. The optimal

temperature of α-amylase was also reported to increase from 30 ºC to 50 ºC after immobilization (Beyler-Cigil *et al*., 2013). Arica *et al*., (1996) reported that when the enzyme is immobilized, the conformational flexibility of its catalytic activity is impaired and higher temperature is required to attain proper structure conformation for its catalytic function. Furthermore, the immobilization of enzyme might alter the conformation of enzyme structure in such a way that the decrease in optimum temperature is also observed (Caramori and Fernandes, 2004). Above 70°C, soluble endo-1, 4-β-D-glucanase lost all its activity, but on the other hand, agar-agar entrapped endo-1, 4-β-D-glucanase retains 67.0 % of its initial activity even at 80°C (Figure 3A). Therefore, it can be suggested that the tertiary structure configuration of entrapped enzyme was protected from high temperature denaturation which might be useful for various industrial process.

Influence of pH on the relative activity of soluble and immobilized enzyme was studied and it was found that the optimal pH-6.0 of the enzyme was unaffected after immobilization (Figure 3B). However, the curves of the pH profile became broader in case of agar-agar entrapment. It was reported that pH maxima of soluble and immobilized cellulase remain the same, i.e. pH-6.0 (Andriani *et al*., 2012). Costa *et al.,* (2001) reported that during the immobilization of enzyme with a polyanionic matrix shifts the optimum pH towards alkaline and if the matrix is polycationic than pH shift is towards acidic. However, our finding suggested that the charge on matrix had no effect on the active site of immobilized endo-1, 4-β-D-glucanase; as the results the optimal pH of immobilized enzyme remains the unchanged. The immobilized enzyme shows higher relativity activity with reference to the soluble enzyme in acidic as well as alkaline conditions. Therefore, it can be inferred that the immobilization within the matrix effectively protects the enzyme subunit dissociation from protonation and de-protonation.

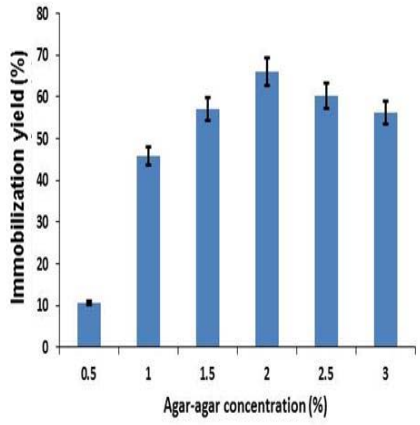

Figure 2: Effect of various agar-agar concentrations on immobilization yield

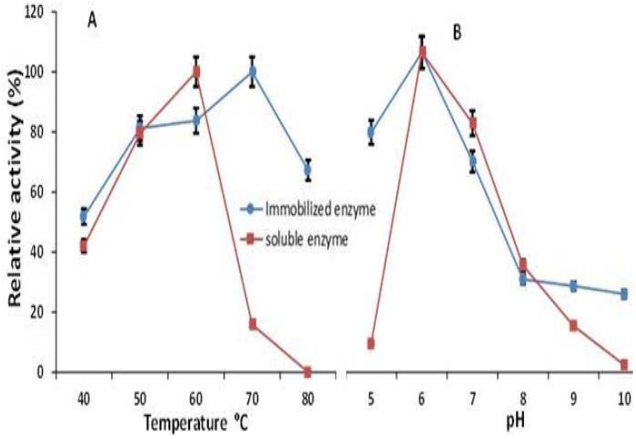

Figure 3: Effect of temperature (A) and pH (B) on soluble and immobilized enzyme activity

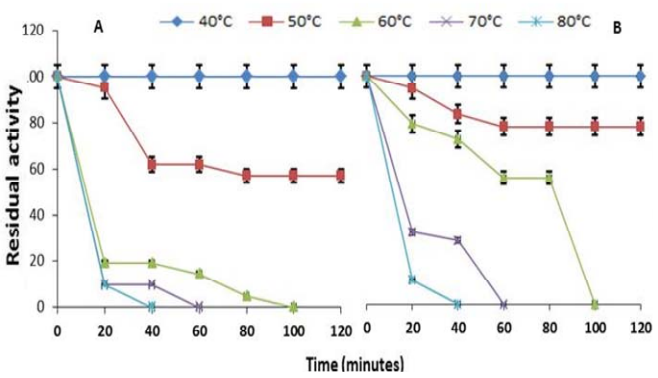

Figure 4: Thermal stability of soluble (A) and immobilized (B) enzyme at various temperatures for varying time periods

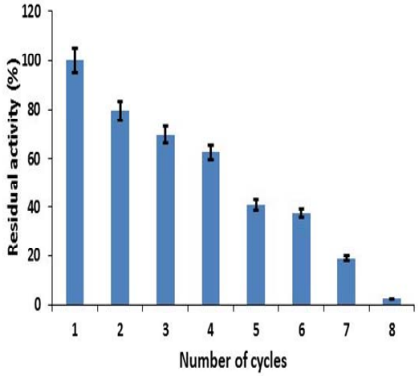

Figure 5: Reusability of immobilized enzyme

3.3 Thermal stability

Thermal stability is one of the important objectives desired as output from immobilization technology and it is a major parameter for the application of enzyme for various industrial processes. In this study, immobilized enzyme showed a significant increase in thermal stability compared to the soluble enzyme (Figure 4). Soluble and immobilized enzyme was found to be 100% stable at 40 °C for 120 minutes. It was observed that incubation of soluble and immobilized enzyme above 40°C, results in reduction of enzyme activity. However, immobilized enzyme activity was reduced at a much slower rate as compare to soluble enzyme. For example, at 50°C, immobilized enzyme retained its activity approximately 1.2 fold higher compared to soluble enzyme, after 120 minutes. Similarly, at 60°C, immobilized enzyme retained approximately 11.1 fold higher activity upon 80 minutes incubation, with reference to soluble enzyme. Moreover, sufficient activity was present in immobilized enzyme at 70°C and 80°C, respectively. Therefore, it is reasonable to assume that immobilization helps in enhancing the thermal stability of enzyme by increasing structures rigidity, which prevents the tertiary configuration of protein from different environmental factors (Wang, *et al*. 2010).

3.4 Kinetic parameters

The K_m and V_{max} values were determined using Lineweaver–Burk plot by varying CMC concentration at constant pH and temperature. It was observed that due to the immobilization, the K_m value was increased from 1.298 mg ml^{-1} to 1.330 mg ml^{-1} and V_{max} was decreased from 8361 U min^{-1} to 470.0 U min^{-1}. These results suggested that a higher concentration of substrate was required for an immobilized enzyme to attain a maximum reaction rate as compared to soluble enzyme. The increase in K_m and the decrease in V_{max} might be due to hindrance in diffusion of high molecular weight substrate (CMC) from bulk to microenvironment (reaction site).

3.5 Reusability of immobilized enzyme

In the current study, immobilized enzyme reusability is given by repeated batch process (Figure 5). The immobilized endo-1, 4-β-D-glucanase retained more than 12% residual activity after 8^{th} cycles. After, 8^{th} cycle the agar-agar tablets became fragile and susceptible to damage. It was also observed that after each cycle enzyme residual activity was decreased, which might be due to inhibition by product, loss of matrix operational stability and leaching of enzyme from agar-agar matrix during continuous use.

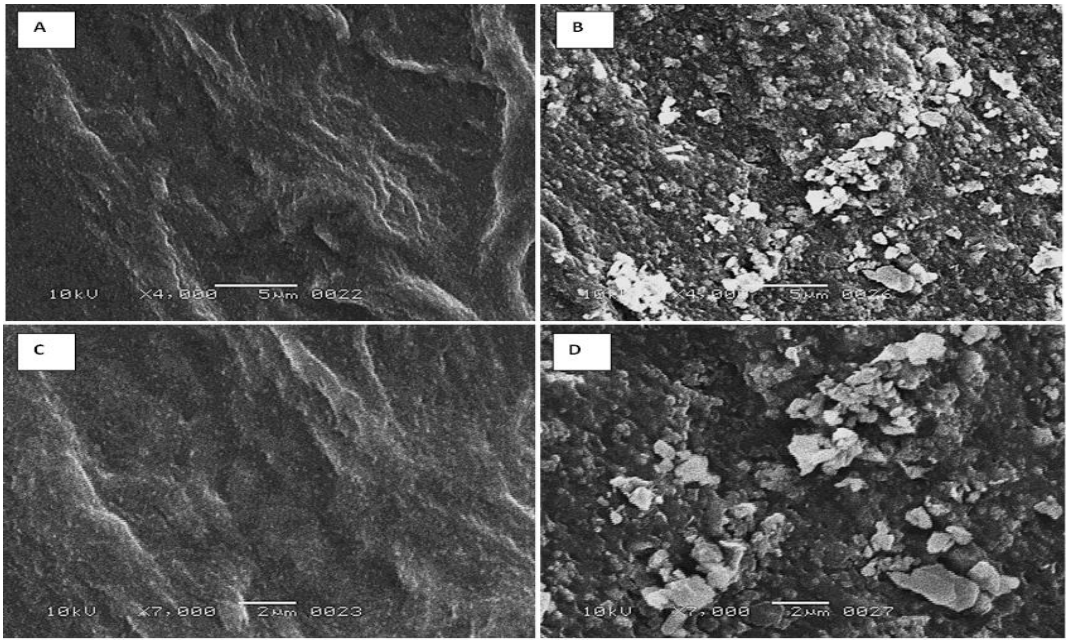

Figure 6: SEM of agar-agar tablets at magnification scales of ×4,000 and ×7,000. SEM images before immobilization (6A) and (6C) and after immobilization (6B) and (6D)

3.6 Scanning electron microscopy

The SEM was used for the study of surface morphologies of agar-agar tables before and after immobilization. In order to study any alteration in the morphology, high resolution image was taken at different magnifications (Figure 6). It was noted that after immobilization the surface morphology of agar-agar was clearly changed and rough surface appears on the surface. On the other hand, the surface of agar-agar before immobilization is having groove and homogenous organize structure. The visualization of surface SEM images of the agar- agar specimens with and without enzyme provided evidence that significant changes occurred in the specimen's surface morphology due to entrapment of an enzyme.

4. Conclusion

Cellulose degrading endo-1, 4-β-D-glucanase by *Bacillus licheniformis* KIBGE-IB2 was immobilized in agar-agar support. As compared to soluble enzyme, the immobilized enzyme showed improved catalytic activity in a wide range of pH and temperature with recycled capabilities. These results indicate that immobilization of endo-1, 4-β-D-glucanase from *Bacillus licheniformis* KIBGE-IB2 in agar-agar support might be used in various industrial processes to improve the product quality as well as decrease the cost of the final product.

References

Afsahi B., Kazemi A., Kheirolomoom A., Nejati S., 2007, Immobilization of cellulase on non-porous ultrafine silica particles, Sci. Iran. 14, 379-383.

Andriani D., Sunwoo C., Ryu H.W., Prasetya B., Park D.H., 2012, Immobilization of cellulase from newly isolated strain *Bacillus subtilis* TD6 using calcium alginate as a support material, Biopr. Biosyst. Eng. 35, 29-33.

Arica M.Y., Alaeddinoglu N.G., Hasirei V., 1996, Immobilization of glucoamylase onto activated PHEMA/EDGMA microspheres: properties and applications into a packed bed reactor, Enzyme Microb. Technol. 18, 281-285.

Beilen J.B.V., Li Z., 2002, Enzyme technology: an overview, Curr. Open. Biotechnol. 13, 338-344.

Beyler-Cigil A., Cakmakci E., Danis O., Demir S., Kahraman M.V., 2013, Alpha-amylase immobilization on modified Polyimide material, Chem. Eng. Trans. 32, 1687-1692.

Bhat M.K., 2000, Cellulases and related enzymes in biotechnology, Biotechnol. Adv. 18, 355-383.

Busto M. D., Ortega N., Perez-Mateos M., 1997, Stabilisation of cellulases by cross-linking with glutaraldehyde and soil humates, Bioresour. Technol. 60, 27-33.

Caramori S.S., Fernandes K. F., 2004, Covalent immobilization of horseradish peroxidase onto poly (ethylene terephthalate) –poly (aniline) composite, Process. Biochem. 39, 883-888.

Costa S.A., Tzanov T., Paar A., Gudelj M., Gübitz G.M., Cavaco-Paulo A., 2001, Immobilization of catalases from Bacillus SF on alumina for the treatment of textile bleaching effluents, Enzyme Microb. Technol. *28*, 815-819.

Gusakov A.V., Berlin A.G., Popova N.N., Okunev O.N., Sinitsyna O.A., Sinitsyn A.P., 2000, A comparative study of different cellulase preparations in the enzymatic treatment of cotton fabrics, Appl. Biochem. Biotechnol. 88, 119-126.

Hildén L., Väljamäe P., Johansson G., 2005, Surface character of pulp fibres studied using endoglucanases, J. Biotechnol. 118, 386-397.

Karnchanatat A., Petsom A., Sangvanich P., Piapukiew J., Whalley A.J., Reynolds C.D., Gadd G.M., Sihanonth P., 2008, A novel thermostable endoglucanase from the wood-decaying fungus *Daldinia eschscholzii* Ehrenb.: Fr. Rehm, Enzyme Microb. Technol. 42, 404-413.

Mateo C., Grazu V., Palomo J.M., Lopez-Gallego F., Fernandez-Lafuente R., Guisan J.M., 2007, Immobilization of enzymes on heterofunctional epoxy supports, Nature Protocols. 2, 1022-1033.

Miller G.L., 1959, Use of dinitrosalicylic acid reagent for determination reducing sugar, Anal. Chem. 31, 426-428.

Ohlson I., Trägårdh G., Hahn-Hägerdal B., 1984, Enzymatic hydrolysis of Sodium hydroxide pretreated sallow in an ultrafiltration membrane reactor, Biotechnol. Bioeng. 26, 647-653.

Tjerneld F., Persson I., Lee J.M., 1991, Enzymatic cellulose hydrolysis in an attrition bioreactor combined with an aqueous two-phase system, Biotechnol. Bioeng. 37, 876-882.

Wang F., Su R., Qi W., Zhang M., He Z., 2010, Preparation and activity of bubbling-immobilized cellobiase within chitosan-alginate composite, Prep. Biochem. Biotechnol. 40, 57-64.

Yuan X.Y., Shen N.X., Sheng J., Wei X., 1999, Immobilization of cellulase using acrylamide grafted acrylonitrile copolymer membranes, J. Membr. Sci. 155, 101-106.