Thermal Characterization of Purified Glucose Oxidase from A Newly Isolated Aspergillus Niger UAF-1

Muhammad Anjum Zia¹, Khalil-ur-Rahman¹, Muhammad K. Saeed²*, Fozia Andaleeb³, Muhammad I. Rajoka³, Munir A. Sheikh¹, Iftikhar A. Khan¹, and Azeem I. Khan¹

¹Department of Chemistry (Biochemistry), University of Agriculture, Faisalabad, Pakistan
²Department of Chemistry, School of Life Science, Beijing Institute of Technology, Beijing 10081, China
³National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan
⁴Centre for Agricultural Biochemistry and Biotechnology, University of Agriculture, Faisalabad, Pakistan

Received 9 November, 2006; Accepted 29 January, 2007

Summary  An intracellular glucose oxidase was isolated from the mycelium extract of a locally isolated strain of Aspergillus niger UAF-1. The enzyme was purified to a yield of 28.43% and specific activity of 135 U mg⁻¹ through ammonium sulfate precipitation, anion exchange and gel filtration chromatography. The enzyme showed high affinity for D-glucose with a Km value of 2.56 mM. The enzyme exhibited optimum catalytic activity at pH 5.5. Temperature optimum for glucose oxidase, catalyzed D-glucose oxidation was 40°C. The enzyme showed a high thermostability having a half-life 30 min, enthalpy of denaturation 99.66 kJ mol⁻¹ and free energy of denaturation 103.63 kJ mol⁻¹. These characteristics suggest the use of glucose oxidase from Aspergillus niger UAF-1 as an analytical reagent and in the design of biosensors for clinical, biochemical and diagnostic assays.

Key Words: glucose oxidase, Aspergillus niger, isolation, purification, kinetics, thermodynamics

Introduction

Glucose oxidase (GOD, β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4) is an important enzyme, which catalyzes the oxidation of β-D-glucose to D-glucono-1, 5-lactone and hydrogen peroxide and finally to gluconic acid using molecular oxygen as electron acceptor [1]. It is widely used in the removal of traces of oxygen and/or glucose from different foods such as dried eggs, beer, wine and fruit juices, as a source of hydrogen peroxide in food preservation and in gluconic acid production [2]. Clinical applications of glucose oxidase in diagnostic tests are likely to be most promising of its applications. The catalytic properties of glucose oxidase form the basis of assaying body fluids such as blood and urine for glucose. A new application for glucose oxidase is its use in biosensors for the determination of glucose [3, 4].

The mycelia fungi Aspergillus niger, Penicillium amagasakiense and P. notatum serve as industrial producers of glucose oxidase. The carbon sources employed in the production of glucose oxidase from Aspergillus niger are mainly glucose and sucrose. The use of cheaper carbon sources appears to be essential for the improvement of process economy [5]. It is generally accepted that the suitability of an enzyme for practical purposes depends on its thermal stability and stability in various media [6]. Thermodynamic parameters are also important for inactivation studies of enzymes [7]. In order to exploit new industrial potentials of glucose oxidase, it is necessary to investigate new microbial strains and to understand the structure-stability relationship of this important enzyme. In this manuscript, we
describe the kinetics and thermodynamics of irreversible thermal inactivation of glucose oxidase from a newly isolated *A. niger* UAF-1 strain grown on corn steep liquor.

**Materials and Methods**

*Organism and Inoculum preparation*

Pure culture of *Aspergillus niger* was obtained from National Fungal Culture Collection of Pakistan (NFCCP), Department of Plant Pathology, University of Agriculture, Faisalabad. It was maintained on potato dextrose agar (PDA) slants at 4°C. Inoculum was prepared by transferring spores from 5–6 days old slant culture, into 250 mL Erlenmeyer flask containing 50 mL of sterile Vogel medium. The flasks were incubated in rotary shaker operating at 150 rpm for 36 hours according to Lineweaver-Burk plot to determine the values of kinetic constants (Vmax and Km).

Production of glucose oxidase

The basal salt medium contained, corn steep liquor 2% (w/v) as substrate along with glucose 2, urea 0.3, CaCO₃ 0.05 and KH₂PO₄ 0.04% to achieve higher glucose oxidase yield using submerged fermentation. Experiments were carried out at initial pH 5.5 and 30°C, unless CaCO₃ was added in the growth medium (resulting in an initial pH 6.5 to 6.8). The flasks were inoculated with 5 mL of pure culture and incubated in rotary shaker operating at 150 rpm for 36 hours for glucose oxidase production [9]. Various parameters were optimized to obtain the maximum yield of the enzyme glucose oxidase which were substrate concentration, pH, temperature, fermentation period, urea, KH₂PO₄, CaCO₃, MgSO₄.7H₂O and glucose. All the experiments were conducted in triplicate and the results were expressed as mean ± standard deviation.

**Analytical**

Total proteins were estimated by Biuret method [10], using bovine serum albumin as the standard. Glucose oxidase activity was determined with the help of a coupled o-dianisidine-peroxidase reaction as described by Worthington *et al.* [11].

Isolation and purification of glucose oxidase

After fermentation mycelia were separated from the culture liquid by filtration and suspended in 0.1 M phosphate buffer (pH 6) and finally disrupted in a homogenizer [12]. The crude extract (200 mL) was subjected to 60–85% saturation with ammonium sulfate saturation [13]. After 24 hours the resulting precipitate was collected by centrifugation at 10,000 rpm for 20 minutes. Desalted sample was applied on DEAE-cellulose column (2.5 × 25 cm) equilibrated with 0.1 M phosphate buffer pH 6. The active fractions were pooled and then applied to sephadex G-150 column (2.5 × 25 cm). Glucose oxidase eluted as a single peak from gel filtration column.

**SDS-PAGE and molecular mass determination**

Finally, the enzyme was subjected to SDS-PAGE 10% to analyze the purity [14]. Standard proteins along with studied enzyme of 1 mg mL⁻¹ each, was loaded on sephadex G-150 column and eluted with 0.1 M phosphate buffer, pH 6 to determine native molecular mass [15].

Effect of pH, temperature, activation energy and substrate

Effect of pH on glucose oxidase activity was determined by assaying the enzyme as mentioned before with the difference that the activity was determined at different pH ranging from 2–9 and in various buffer solutions as described earlier [16]. Temperatures ranging from 20–70°C, activation energy was determined from the Arrhenius plot [17] and it was assayed in the reaction mixtures containing variable amounts of glucose i.e. 5–40 mM) at pH 5.5. The data was analyzed according to Lineweaver-Burk plot to determine the values of kinetic constants (Vmax and Km).

Kinetics of thermal denaturation

Kinetic and thermodynamic parameters for irreversible thermal denaturation of glucose oxidase were determined by incubating the enzyme in 50 mM MES monohydrate buffer (pH 5.5) at a particular temperature. Aliquots were withdrawn at different times, cooled on ice for 3 h [18] and assayed for enzyme activity at 25°C. This procedure was repeated at five different temperatures ranging from 45 to 60°C. The data were fitted to first order plots (Fig. 4) and analyzed as described earlier [19]. The thermodynamic parameters for thermostability were calculated by rearranging the Eyring’s absolute rate equation derived from the transition state theory as described by Siddiqui *et al.* [20].

\[
kd = (k_b T/h)e^{(-\Delta H^*/RT)}e^{(\Delta S^*/R)} .................................................. (1)
\]

Where,

\[
h = \text{Planck’s constant} = 6.63 \times 10^{-34} \text{ Js}
\]

\[
k_b = \text{Boltzman’s constant (R/N)} = 1.38 \times 10^{-23} \text{ JK}^{-1}
\]

\[
R = \text{gas constant} = 8.314 \text{ JK}^{-1} \text{ mol}^{-1}
\]

\[
N = \text{Avogadro’s No.} = 6.02 \times 10^{23} \text{ mol}^{-1}
\]

\[
T = \text{Absolute temperature}
\]

\[
\Delta H^* (\text{enthalpy of activation}) = \text{Ea} - \text{RT} .................................. (2)
\]

\[
\Delta G^* (\text{free energy of activation}) = -\text{RT ln (k_a/h/k_bT)} ...... (3)
\]

\[
\Delta S^* (\text{entropy of activation}) = (\Delta H^* - \Delta G^*)/T .................. (4)
\]
Results and Discussion

Production of Glucose oxidase

In the present studies, corn steep liquor (CSL) was used for growth and glucose oxidase production by the *Aspergillus niger* UAF-1. Culture media containing 2% substrate was subjected to fermentation for 36 hours at pH 5.5, 30°C temperature and 5% inoculum size. The results indicated enzyme activity (6.06 U mL$^{-1}$) in a medium containing 2% CSL. The findings of present study are in accordance with most of the research workers [21, 22]. For the optimization of substrate, growth media containing 2% corn steep liquor was found to be the best by Zareen [23]. Furthermore, no studies have been found about the utilization of corn steep liquor as a substrate/inducer for glucose oxidase production. So, we utilized CSL as a substrate for the said enzyme production to optimize the conditions of better production and to explore the application of CSL as substrate for glucose oxidase. Submerged fermentation was carried out for 24, 36, 48, 60 and 72 hours with 2% CSL. Enzyme obtained high activity i.e. 7.88 U mL$^{-1}$, after 36 hours incubation, thereafter biosynthesis of enzyme decreased. Willis [24] optimized the fermentation media for the production of glucose oxidase by *Aspergillus niger* and obtained highest glucose oxidase yield after 48 h. While our results indicate the maximum production of the enzyme after 36 h. Media was supplemented with glucose as carbon additive and fermentation carried out under optimum conditions. Addition of 4% glucose to fermentation flasks (in triplicate) resulted in better glucose oxidase production with 23.74 U mL$^{-1}$ activity. Willis [24] optimized the fermentation media for the production of glucose oxidase by *Aspergillus niger* and obtained highest glucose oxidase yield after 48 h. While our results indicate the maximum production of the enzyme after 36 h. Media was supplemented with glucose as carbon additive and fermentation carried out under optimum conditions. Addition of 4% glucose to fermentation flasks (in triplicate) resulted in better glucose oxidase production with 23.74 U mL$^{-1}$ activity. It was observed after optimization of media that 2% substrate, 36 h of fermentation, pH 5.5, temperature 30°C, urea 0.3%, KH$_2$PO$_4$ 0.6%, CaCO$_3$ 0.04% and glucose 4% proved to be best, while with addition of MgSO$_4$.7H$_2$O production of enzyme decreased [26, 27] (Fig. 1).

Purification of glucose oxidase

An intracellular glucose oxidase was purified from the culture of *Aspergillus niger* UAF-1 strain grown in submerged corn steep liquor medium. Disintegration of the mycelia resulted in a very high glucose oxidase activity in the mycelium extract. The specific activity of crude extract was 17.98 U mg$^{-1}$ protein. The complete precipitation of the enzyme was observed at 85% ammonium sulfate. Purification of the enzyme on anion exchange column was 3.03 fold with 50.42% recovery. Sukhacheva et al. [15] subjected the *P. funiculosum*-433 glucose oxidase to 80% saturation and found 18 U mg$^{-1}$ specific activity with 94% yield and 1.6 fold purification and it was applied to DEAE-cellulose column, resulted the decrease in protein contents, increased recovery of 56.2%. On gel filtration column, the enzyme was purified to 7.5 fold, specific activity of 135 U mg$^{-1}$ with a yield of 28.43%. Liu et al. [28] pooled the fractions from DEAE-Sepharose and subjected to gel filtration, observed 36% recovery and 36 fold enrichment of the enzyme. These results are in accordance to this study, as indicated in Table 1.

SDS-PAGE and molecular mass determination

SDS-PAGE (10%) upon treatment of the purified enzyme with mercaptoethanol demonstrated a single band with a...
Glucose Oxidase from *Aspergillus Niger* UAF-1

Table 1. Summary of purification of *Aspergillus niger* UAF-1 glucose oxidase

| Treatment                                | Total Activity (U) | Total protein (mg) | Specific activity (U mg⁻¹) | Purification fold | % Recovery |
|------------------------------------------|--------------------|--------------------|----------------------------|-------------------|------------|
| Crude                                    | 2374               | 132                | 17.98                      | 1                 | 100        |
| Ammonium sulfate precipitation           | 1589               | 74                 | 21.47                      | 1.19              | 66.93      |
| Anion exchange chromatography            | 1197               | 22                 | 54.41                      | 3.03              | 50.42      |
| Gel filtration chromatography            | 675                | 5                  | 135                        | 7.5               | 28.43      |

mobility corresponding to a molecular weight of 70 kDa. This indicated that the enzyme is formed of two identical subunits. The native and subunit molecular masses of glucose oxidase from *Aspergillus niger* UAF-1, have been determined on sephadex G-150 column and SDS-PAGE, which were found as 175 kDa and 170 kDa, respectively, Ferreira et al. [29] found that molecular mass of *A. niger* glucose oxidase is of 160 kDa and this finding is also supported by Zoldak et al. [30].

**Effect of pH, temperature activation energy and Effect of substrate**

Glucose oxidase from *Aspergillus niger* UAF-1 was active within the pH range of 4–8, while maximum activity was observed at pH 5.5 (Fig. 2). The activation energy (Ea) and optimum temperature of glucose oxidase was found to be 15.46 kJ mol⁻¹ and 40°C respectively. It is obvious from the Arrhenius plot (Fig. 3) that the enzyme had a single conformation up to transition temperature. The change in pH affects the ionization of essential active site amino acid residues, which are involved in substrate binding and catalysis. The ionization of these residues may cause distortion of active site cleft and hence indirectly affect the enzyme activity. Our results favorably compare to those of Weibel and Bright [31], who reported that glucose oxidase is working in the pH range of 4–7 indicating 5.5 as optimum. *Aspergillus niger* UAF-1 enzyme showed a low Ea at 40°C that makes the enzyme superior to the enzyme from various other sources. Purified glucose oxidase having a protein content of 0.05 mg mL⁻¹ was used for the kinetic and thermodynamic characterization. The Km and Vmax values obtained from Lineweaver-Burk plot were 2.56 mM and 43.5 U mg⁻¹ protein (Fig. 4). Referring to properties of biotechnological relevance, the glucose oxidase of *Aspergillus niger* exhibited a high affinity for D-glucose as it has low Km value for the substrate as compared to a high Km value (0.033 M) of glucose oxidase isolated from the *Aspergillus niger* reported earlier [32]. This high substrate

![Fig. 2. Effect of pH on activity of *Aspergillus niger* UAF-1 glucose oxidase](image1)

![Fig. 3. Arrhenius plot for determination of activation energy for the oxidation of D-glucose.](image2)

![Fig. 4. Double reciprocal plot to determine the kinetic constants for D-glucose](image3)
affinity and specificity, in addition to its long-term stability in the pH range 4–8, proved glucose oxidase of *Aspergillus niger* UAF-1 as a suitable biocatalyst for industrial applications. The rates of reaction of glucose oxidase from *Aspergillus niger* with glucose, 2-deoxyglucose, mannose, galactose and xylose have been measured and found that glucose reacts much faster than the other sugars with $K_m$ 0.11 M and $V_{\text{max}}$ of 2000 s$^{-1}$ M$^3$3).

**Thermal denaturation studies**

Glucose oxidase from *Aspergillus niger* UAF-1 was thermally stable at 45°C with half-life of 173 minutes. However at 60°C it was less stable and displayed a half-life of 30 min under similar conditions (Fig. 5). The enzyme had a range of 99.79–99.66 kJ mol$^{-1}$ enthalpy of denaturation ($\Delta H^*$) at 45°C–60°C. The value of free energy of thermal denaturation ($\Delta G^*$) for glucose oxidase was 103.47 kJ mol$^{-1}$ at 45°C, showing a decreasing trend with increase in temperature.

When entropy of inactivation ($\Delta S^*$) was calculated at each temperature, it showed negative values. Purified glucose oxidase from *Aspergillus niger* UAF-1 showed a $\Delta S^*$ value of −11.92 J mol$^{-1}$ K$^{-1}$ at 60°C (Fig. 6, Table 2).

Thermostability is the ability of enzyme to resist against thermal unfolding in the absence of substrates, while thermophilicity is the capability of enzymes to work at elevated temperatures in the presence of substrate [34]. Purified glucose oxidase from *Aspergillus niger* UAF-1 showed appreciable stability and thermodynamic characteristics between 45–60°C. Thermal denaturation may occur in two steps as shown:

$$\text{N} \leftrightarrow \text{U} \rightarrow \text{D}$$

Where, N (native enzyme), U (unfolded enzyme) that could be reversibly refolded upon cooling and D is the denatured enzyme formed after prolonged exposure to heat and therefore cannot be recovered on cooling. Glucose oxidase isolated from this *Aspergillus niger* strain was thermally stable and could be used for analytical and other industrial applications. The negative entropy of deactivation observed for glucose oxidase suggested that there was negligible disorderliness as that of β-glucosidase from *Aspergillus wentii* or the transition state of α-amylase from *Bacillus licheniformis* was found to be more ordered as revealed by its negative $\Delta S^*$ at high temperature of 80°C. According to [35] the values 81.8 kcal mol$^{-1}$, 24.6 kcal mol$^{-1}$ and 169.2 cal K$^{-1}$ mol$^{-1}$ of $H^*$, $G^*$ and $S^*$ respectively, were obtained for standard *Aspergillus niger* glucose oxidase. A high value for free energy of thermal denaturation at 60°C indicated that glucose oxidase exhibited the resistance against thermal unfolding at higher temperatures. Gouda et al. [36] reported that *Aspergillus niger* glucose oxidase has an activation

| Temp. (K) | $k_d$ (min$^{-1}$) | $t_1/2$ (min) | $\Delta H^*$ kJ mol$^{-1}$ | $\Delta G^*$ kJ mol$^{-1}$ | $\Delta S^*$ J mol$^{-1}$ K$^{-1}$ |
|-----------|-------------------|--------------|-----------------|-----------------|-----------------|
| 318       | 0.004             | 173          | 99.79           | 103.47          | −11.57          |
| 321       | 0.007             | 99           | 99.76           | 102.98          | −10.03          |
| 325       | 0.013             | 58           | 99.73           | 102.84          | −9.57           |
| 329       | 0.018             | 39           | 99.71           | 103.03          | −10.12          |
| 333       | 0.023             | 30           | 99.66           | 103.63          | −11.92          |
Glucose Oxidase from *Aspergillus Niger* UAF-1

Table 3. Comparison of various characteristics of purified glucose oxidase from *Aspergillus niger* UAF-1 with other sources

| Characteristics                          | *A. niger* UAF-1 Glucose oxidase | Glucose oxidase from other sources |
|------------------------------------------|----------------------------------|-----------------------------------|
| Activity enzyme (U)                      | 675                              | NA                                |
| Specific activity (U mg⁻¹)               | 135                              | NA                                |
| % age Yield                              | 28.43                            | 36°                               |
| Optimum pH                               | 5.5                              | 4–7°                              |
| Optimum temperature (°C)                 | 40                               | >40°                              |
| Eₐ (kJ mol⁻¹)                            | 15.46                            | NA                                |
| Kₘ (mM)                                  | 2.56                             | 5.7c                              |
| Vₘₙ (U mg⁻¹)                             | 43.5                             | 925c                              |
| Molecular wt. (kDa)                      | 170                              | 160d                              |
| k₉ (min⁻¹) at (60°C)                     | 0.023*                           | NA                                |
| t½ (min)                                 | 30°                             | NA                                |
| ΔH* (kJ mol⁻¹)                           | 99.66°                           | NA                                |
| ΔG* (kJ mol⁻¹)                           | 103.63°                          | 88.3 kcal mol⁻¹                   |
| ΔS* (J mol⁻¹ K⁻¹)                        | -11.92°                          | 184 cal. mol⁻¹                    |

NA = Not reported

Glucose oxidase from *Aspergillus niger* (Liu et al. 1998)
Glucose oxidase from *Penicillium pinophilum* (Rando et al. 1997)
Glucose oxidase from genetically modified *P. amagasakiense* (Witt et al. 1998)
Glucose oxidase from *Aspergillus niger* (Ferreira et al., 2005).
Results obtained at 60°C.

*Conclusions*

Glucose oxidase was isolated from the mycelium extract of a locally isolated strain of *A. niger* UAF-1. It was purified by using ammonium sulfate precipitation and chromatographic techniques and its yield and specific activity was 28.43%, 135 U mg⁻¹ respectively. The enzyme showed high affinity for D-glucose with a Km value of 2.56 mM, exhibited optimum catalytic activity at pH 5.5 and optimum temperature for glucose oxidase, catalyzed D-glucose oxidation was 40°C. It showed a high thermostability having a half-life of 30 min, enthalpy of denaturation 99.66 kJ mol⁻¹ and free energy of denaturation 103.63 kJ mol⁻¹. These characteristics suggest the use of glucose oxidase from *Aspergillus niger* UAF-1 as an analytical reagent and in the design of biosensors for clinical, biochemical and diagnostic assays. However further studies are required to elucidate the broader implication of this enzyme.

**References**

[1] Betancol, L., López-Gallego, F., Hidalgo, A., Alonso-Morales, N., Dellamora-Ortiz, G., Guisán, J.M., and Fernández-Lafuente, R.: Preparation of a very stable immobilized biocatalyst of glucose oxidase from *Aspergillus niger*. *J. Biotechnol.*, 121, 284–289, 2006.
[2] Anastassiadis, S., Aivasidis, A., and Wandrey, C.: Continuous gluconic acid production by isolated yeast-like mould strains of *Aureobasidium pullulans*. *Appl. Microbiol. Biotechnol.*, 61, 110–117, 2003.
[3] Malhotra, B.D., Singhal, R., Chauhey, A., Sharma, S.K., and Kumar, A.: Recent trends in biosensors. *Curr. Appl. Phys.*, 5, 92–97, 2005.
[4] Sunga, W.J. and Baeb, Y.H.: Glucose oxidase, lactate oxidase, and galactose oxidase enzyme electrode based on polypropylene with polyanion/PEG/enzyme conjugate dopant. *Sensors and Actuators B*, 114, 164–169, 2006.
[5] Tellez-Iurado, A., Arana-Cuenca, A., Gonzalez, B.A.E., Viniegra-Gonzalez, G., and Loera, O.: Expression of a heterologous laccase by *Aspergillus niger* cultured by solid-state and submerged fermentations. *Enzyme Microb. Technol.*, 38, 665–669, 2006.
[6] Vasilevaa, N. and Godjevargova, T.: Study of the effect of some organic solvents on the activity and stability of glucose oxidase. *Mat. Sci. Engineer.*, 25, 17–21, 2005.
[7] Iqbal, Z., Rashid, M.H., Jabar, A., Malana, M.A., Khalid, A.M., and Rajoka, M.I.: Kinetics of enhanced thermostability of an extracellular glucomylase from *Arachniotus* sp. *Biotechnol. Let.*, 25, 1667–1670, 2003.
[8] Haq, I., Khurshid, S., Ali, S., Ashraf, H., Qadeer, M.A., and Rajoka, M.I.: Mutation of *Aspergillus niger* hyperproduction of citric acid from black strap molasses. *World J. Microbiol. Biotechnol.*, 17, 35–37, 2001.
[9] Hamid, H.M., Rehman, K., Zia, M.A., and Asghar, M.: Optimization of various parameters for the glucose oxidase from rice polishing using *Aspergillus niger*. *Biotechnol.*, 2, 1–7, 2003.
[10] Gornall, A.G., Bardwill, C.J., and David, M.M.: Determination of serum proteins by means of biuret reagent. *J. Biol. Chem.*, 177, 751–766, 1949.
[11] Worthington, C.C.: Worthington enzyme manual: enzymes and related biochemical. Worthington Biochem. Coop., USA, pp. 155–158, 254–260, 1988.
[12] Hatzinikolaou, D.G. and Macris, B.J.: Factors regulating production of glucose oxidase by *Aspergillus niger*. *Enzyme Microbial. Technol.*, 17, 530–534, 1995.
[13] Shin, K.S., Youn, H.D., Han, Y.H., Kang, S.O., and Hah, Y.C.: Purification and characterization of D-glucose oxidase from white-rot fungus *Pleurotus ostreatus*. *Euro. J. Biochem.*, 215: 747–752, 1993.
[14] Kotik, M. and Kyslík, P.: Purification and characterization of a novel enantioselective epoxide hydrolase from *Aspergillus niger* M200. *Biochem. Biophys. Acta*, 1760, 245–252, 2006.
[15] Sukhacheva, M.V., Davydova, M.E., and Netrusov, A.I.: Production of *Penicillium funiculosum* 433 glucose oxidase and its properties. *Appl. Biochem. Microbiol.*, 40, 25–29.
Tzonka, G., Nenkova, R., and Konsulov, V.: Immobilization of glucose oxidase by acrylonitrile copolymer coated silica supports. *J. Mol. Catal. B Enzym.*, 38, 59–64, 2006.

Rashid, M.H. and Siddiqui, K.S.: Thermodynamic and kinetic study of stability of the native and chemically modified β-glucosidase from *Aspergillus niger*. *Process Biochem.*, 33, 109–115, 1998.

Violet, M. and Munier, J.C.: Kinetic study of irreversible thermal denaturation of *Bacillus licheniformis* α-amylase. *Biocatalyst and Biotransformation*, 163, 665–670, 1989.

Montes, F.J., Battanar, E., Catalan, J., and Galan, M.A.: Kinetics and heat inactivation mechanism of D-amino oxidase. *Process Biochem.*, 30, 217–224, 1995.

Siddiqui, K.S., Shamsi, A.M., Anwar, M.A., Rashid, M.H., and Rajoka, M.I.: Partial and complete alternation of surface changes of carboxymethyl cellulase by chemical modification: thermostabilization in water-miscible organic solvent. *Enzyme Microbial Technol.*, 24, 599–608, 1999.

Ellaiah, P., Adinarayana, K., Bhavani, Y., Padmaja, P., and Srinivasulu, B.: Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergillus* species. *Proc. Biochem.*, 38: 615–620, 2002.

Parekh, M., Formanek, J., and Blaschek, H.P.: Pilot scale production of butanol by *Clostridium beijerinckii* BA101 using a low cost corn steep water based fermentation medium. *Appl. Microbiol. Biotechnol.*, 51, 152–157, 1999.

Zareen, N.B.G.: Characterization and purification of glucose oxidase produced from *Aspergillus niger* using corn steep liquor. M.Sc. thesis Department of Chemistry, University of Agriculture, Faisalabad, pp. 30–70, 2004.

Willis, A.W.: Methods in Enzymology. Vol. IX. Academic Press, USA, pp. 83–84, 1966.

Kona, R.P., Qureshi, N., and Pai, J.S.: Production of glucose oxidase using *Aspergillus niger* and corn steep liquor. *Bioresource Technol.*, 78, 123–126, 2001.

Fiedurek, J. and Gromada, A.: Production of catalase and glucose oxidase by *Aspergillus niger* using unconventional oxygenation of culture. *J. Appl. Microbiol.*, 89, 85–89, 2000.