Isolation and characterization of urease from *Aspergillus niger*

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Urease was purified (4126-fold) from *Aspergillus niger* (NRRL 003) to a homologous enzyme preparation with a specific activity of 1341 μmol min⁻¹ (mg protein)⁻¹. One species of urease was detected in *A. niger*, with *Kₐ* = 30 μm, native molecular mass 250000 Da, pH optimum of 8.0 and a high specificity for urea. Hydroxyurea was a strong competitive inhibitor of urease activity, while N-methylurea acted as a weak uncompetitive inhibitor, based on Lineweaver-Burk and Eadie-Hofstee plots. The activity of urease was enhanced by, but not dependent on, the presence of Na,EDTA, DL-dithiothreitol (<0.1 to 5.0 mM), Ca²⁺, Ba²⁺ and citrate (2 to 20 mM). Urease activity was not affected by Na⁺, K⁺, Cl⁻, Br⁻, acetate or nitrate (2 to 20 mM), but was significantly decreased in the presence of Li⁺, Ni²⁺, Mg²⁺, Zn²⁺ or I⁻. Urease activity decreased 26.0% after 30 min at 65 °C, and 86.5% and 100.0% after 5 and 1 min at 80 and 100 °C, respectively. Urease activity decreased 30.5% after 90 d at 4 °C and 21.0% after 28 d at −20 or −80 °C.

Introduction

*Aspergillus niger* has been used for the commercial production of citric acid and cellulolytic enzymes (Friedrich et al., 1989; Gokhale et al., 1991; Lee et al., 1989; Roukas, 1991). The technology for producing large quantities of *A. niger* mycelia has been established, thus alternative uses of *A. niger* are being investigated.

One enzyme of increasing interest is acid urease (urea amidohydrolase, EC 3.5.1.5). Acid ureases obtained from *Lactobacillus* strains have been used to reduce urea levels in wine, thereby preventing the formation of ethyl carbamate, a carcinogen (Famuyiwa & Ough, 1991; Fujinawa et al., 1990; Kakimoto et al., 1989; Ough & Trioli, 1988). However, *Lactobacillus* spp. yield low quantities of urease, and alternative microbial sources of the enzyme are being sought. Urease activity in *A. niger* was first reported in 1903, yet the characteristics of this enzyme have not been determined (Pomar et al., 1970). This strain of *Aspergillus* could be used for the production of an acid urease, for the fungus grows readily under acidic (wine) conditions. Here, we report the first isolation and characterization of urease from *A. niger* and suggest which steps in the purification strategy may be suitable for industrial applications of the purified enzyme.

Methods

**Chemicals.** Materials used in the suspension culture medium were obtained from Difco. Other chemicals, prepared solutions (alkaline hypochlorite and phenol-nitroprusside solutions) and Phenyl Sepha-
rose were obtained from Sigma. DEAE Fast Flow Sepharose and Sephacryl S 300 were from Pharmacia. Macro-Prep Q anion exchange media and BioRad Protein Stain were from BioRad.

**Aspergillus niger culture.** The *A. niger* (NRRL 003) strain used for this experiment was obtained from the USDA culture collection at Peoria, Illinois, USA. Thirty strains of *Aspergillus niger*, *Aspergillus aculeatus* and *Aspergillus ficuum* were screened for the presence of urease. The *Aspergillus* species were grown on maltose extract agar at 25 °C in the dark for 7 d and conidia were harvested with sterile water containing 0.05% Tween 80. The growth media used in the production of *Aspergillus* mycelia contained the following: 20% (w/v) glucose, 1% (w/v) yeast extract, 1% (w/v) yeast nitrogen base without amino acids, 0.5% citrate, 15% (w/v) NaHPO₄, 0.0032% NiSO₄·6H₂O and the pH of the growth media was adjusted to 3.0 with tartaric acid. Filter (0.2 μm) sterilized urea was added after autoclaving to obtain a final urea concentration of 0.02% (or 3 mM). The growth medium (1 litre in a 2.8 l Fernbach flask) was inoculated with a suspension of *Aspergillus* conidia and shaken (250 r.p.m.) at 35 °C for 2 d in an Environ-Shaker (Lab-Line Instruments). *Aspergillus* mycelia were harvested by filtering through four layers of cheesecloth, rinsing thoroughly with water, then immediately freezing at −80 °C. The frozen mycelia were lyophilized, ground to a fine powder in a Wily mill and stored at −80 °C until needed. Based on the levels of urease activity, *Aspergillus niger* (NRRL 003) was selected for further analysis.

**Urease assay.** Urease activity was determined with a modified protocol based on Weatherburn (1967). Urease reactions were done in

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*Abbreviation: HEPPSO, N-2-hydroxyethylpiperazine-N'2-hydroxypropanesulphonic acid.*
1.5 ml microcentrifuge tubes, with a final reaction volume of 1.0 ml containing 100 mm-potassium phosphate buffer (pH 8.0), 50 mm-urea and enzyme extract (5 to 100 μl). Urease reactions were initiated by the addition of the enzyme extract and incubated at 37 °C for 30 min while mixing (700 r.p.m.) on an Eppendorf 5436 Thermomixer (Brinkman). The reaction was stopped by transferring 100 μl of the reaction mixture to a 1.5 ml microcentrifuge tube containing 500 μl of a 50% (w/v) phenol-nitroprusside solution. Five hundred microliters of a 0.2% alkaline hypochlorite solution and 150 μl water were added, the mixture was then shaken, and the color complex was allowed to develop for a minimum of 10 min at room temperature. Ammonia concentrations generated from the urease reaction, represented by the color complex, were determined at 625 nm with a Gilford UV/VIS Spectrophotometer (Ciba Corning Diagnostics) and compared to a standard curve made with NH₄Cl. Controls used for the enzyme reactions were: (a) the reaction mixture at time zero; (b) boiled enzyme extract; and (c) the reaction mixture without urea. The enzyme extracts were regularly diluted before assaying to ensure that the activity determinations were linear with respect to time.

Enzyme purification. All stages of the urease isolation were performed at 4 °C, unless otherwise specified.

(i) Enzyme extraction. Lyophilized and ground A. niger mycelia (35 g) were extracted in 30 vols (1:30, w/v) of 50 mm-potassium phosphate buffer (pH 7.0) in a 2.8 l Fernbach flask for 30 min while shaking (250 r.p.m.) on a Laboratory Rotator Shaker Model G2 (New Brunswick Co.). The homogenate was centrifuged at 25000 g for 20 min, and the clear supernatant was retained for further purification. Urease activity in the crude extract was measured as previously described, and the concentration of soluble protein was determined by the Bradford method (Bradford, 1976). Solid NaCl was added to the crude extract to attain a final NaCl concentration of 200 mm.

(ii) First anion exchange chromatography. Crude extract (950 ml) containing 200 mm-NaCl was transferred to a DEAE Fast Flow Sepharose column (4.5 cm i.d. x 6.0 cm) pre-equilibrated with the running buffer (RB: 50 mm-potassium phosphate, pH 7.0) containing 200 mm-NaCl. The DEAE column was washed at a flow rate of 15 ml min⁻¹ with eight bed vols RB (containing 200 mm-NaCl). Urease activity was eluted from the column with RB containing 350 mm-NaCl. Eluate was partitioned into 5 ml fractions with a Gilson Microfractionator. Fractions with the highest urease activity were pooled and retained for further purification. The practice was repeated with the subsequent columns and was essential for obtaining a pure enzyme preparation, although a significant loss of total activity often resulted.

(iii) Hydrophobic chromatography. Pooled DEAE fractions (50 ml) were transferred to a Phenyl Sepharose column (2.5 cm i.d. x 40 cm), pre-equilibrated with RB containing 350 mm-NaCl. The column was sequentially washed at a flow rate of 50 ml min⁻¹ with ten bed vols RB containing 350 mm-NaCl, then an additional ten bed vols RB. The enzyme was eluted from the column with aqueous ethylene glycol (EG, 50%, v/v) and 2.5 ml fractions were collected as before.

(iv) Second anion exchange chromatography. Pooled Phenyl Sepharose fractions (7 ml) were dialysed overnight at 4 °C against 1 l of running buffer 2 (RB2: 25 mm-potassium phosphate buffer, pH 8.0) containing 100 mm-KCl. The dialysed sample was filtered (0.2 μm filter) and transferred to a fast protein liquid chromatography (Pharmacia) Macro-Prep Q anion exchange column (Macro-Prep column; 1.5 cm i.d. x 1.0 cm) pre-equilibrated with RB2 containing 100 mm-KCl. Washing the Macro-Prep column with higher KCl concentrations permitted part of the urease activity to pass directly through the column. The Macro-Prep column was operated at room temperature. The Macro-Prep column was washed with 4 bed vols (each) of RB2 containing 100 mm-KCl, RB2 containing 180 mm-KCl, and RB2 containing 220 mm-KCl. Urease activity was eluted from the column with an increasing linear KCl gradient (220 to 500 mm) in RB2.

(e) Size exclusion chromatography. The volume (3 ml) of pooled Macro-Prep column fractions was reduced to 500 μl with a Centricon-30 Concentrator (Amicon). The entire concentrated sample was transferred to a Sephacryl S 300 column (S 300 column; 1.5 cm i.d. x 110 cm) pre-equilibrated with RB. The flow rate of the S 300 column was maintained at 0.2 ml min⁻¹ with a Gilson Minipuls 3 Peristaltic Pump. Fractions (1.0 ml) with the highest activity were pooled and retained for the characterization studies. A 200 μl sample of crude extract and each successive purification was set aside and treated for SDS-PAGE using a modified protocol of Laemmli (1970).

Native molecular mass estimation. The native molecular mass of urease was determined using a Sephacryl S 300 column. The purified enzyme extract (300 μl) was applied to the column and eluted at a flow rate of 0.2 ml min⁻¹. The native molecular mass was determined after multiple runs with: (a) purified enzyme; (b) purified enzyme with deswann blue; and (c) purified enzyme and molecular mass standards. The native molecular mass estimation was derived from a regression plot (r = 0.999) of Vᵣ/Vₐ vs log M₀. Vᵣ represented the void volume of the S 300 column, while Vₐ represented the void volume plus the volume eluted from the column with the urease. The molecular mass standards used for this estimation were as follows: thyroglobulin (669 kDa), apoferritin (443 kDa), bovine α-globulin (158 kDa), chicken albumin (44 kDa) and equine myoglobin (17 kDa). Column fractions collected from the S 300 column were assayed for activity, while molecular mass protein standards were detected by the method of Bradford (1976).

Results and Discussion

Enzyme purification

Urease activity was purified 4126-fold from A. niger with the isolation scheme presented in Table 1. This isolation strategy recovered 0.55% of the original activity and achieved a final specific activity of 1341 pmol min⁻¹ (mg protein)⁻¹. The specific activity of the final enzyme preparation was twice that of previous purifications for enzymes of Aspergillus nidulans and Aspergillus tamarii (Creaser & Porter, 1985; Mackay & Pateman, 1980, 1982; Zavada & Sutcliffe, 1981).

The first stage of purification, following extraction of urease from ground lyophilized mycelia, involved a direct application of the crude extract to DEAE column chromatography. Purifications of urease are usually initiated with an acetone, ammonium sulphate or ethanol precipitation (Creaser & Porter, 1985; Evans et al., 1991; Mahadevan et al., 1977; Rai, 1989). Increasing the NaCl concentration to 200 mm in the crude extract prior to DEAE chromatography allowed the retention of all of the urease activity by the column while permitting > 95% of the soluble protein to elute directly through the column. This replaced the initial concentrating or precipitation step. Christians et al. (1991) also began a purification of urease from Staphylococcus xylosus with an anion exchange column, although the salt concentration of the crude extract was not increased before transferring the sample to the column. Elution of > 80% of the urease activity was achieved with the application...
Aspergillus niger urease activity

Table 1. Isolation of urease from Aspergillus niger

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (µmol min⁻¹)</th>
<th>Total protein (mg)</th>
<th>Specific activity [µmol min⁻¹ (mg protein)⁻¹]</th>
<th>Purification (-fold)</th>
<th>Activity recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1066.1</td>
<td>3278.9</td>
<td>0.325</td>
<td>—</td>
<td>100.00</td>
</tr>
<tr>
<td>DEAE Sepharose</td>
<td>361.4</td>
<td>99.863</td>
<td>3.619</td>
<td>11.1</td>
<td>33.90</td>
</tr>
<tr>
<td>Phenyl Sepharose</td>
<td>118.5</td>
<td>12833</td>
<td>9.234</td>
<td>28.4</td>
<td>11.10</td>
</tr>
<tr>
<td>Macro-Prep Q Anion Exchange</td>
<td>24.2</td>
<td>0.0871</td>
<td>277.8</td>
<td>85.48</td>
<td>2.27</td>
</tr>
<tr>
<td>Sephacryl S 300</td>
<td>5.9</td>
<td>0.0044</td>
<td>1340.9</td>
<td>412.58</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Values are means of four purifications.

of RB containing 350 mM-NaCl. The enzyme was eluted from the DEAE column in a single activity peak. Therefore, direct application of the crude A. niger extract to a DEAE column could be suitable for industrial purification of urease. When fractions with the highest urease activity were retained, 33.9% of the original urease activity was recovered from the DEAE column, while eliminating > 97% of the soluble protein in the enzyme preparation (Table 1). If the entire urease activity peak was retained from the DEAE column, the pooled fractions contained > 80% of the original activity with < 10% of the initial soluble protein. Additionally, a single DEAE column was used for all purifications experiments in this study and elution of urease activity from the column was reproducible. Therefore, a highly purified urease preparation can be achieved with a single predictable and reproducible step.

When the pooled DEAE fractions were transferred to the Phenyl Sepharose column, all urease activity was retained by the column. Previously, Phenyl Sepharose (or hydrophobic) columns have been used effectively in the purification of urease (Dunn et al., 1991). Washing the column with RB containing 350 mM-NaCl and RB did not elute detectable urease activity. The 50% EG solution eluted > 99% (or 33% of the original activity) of the urease activity bound to the Phenyl Sepharose column. Of this urease activity, 11.1% (of the original activity) was retained for further purification.

The pooled Phenyl Sepharose fractions were dialysed overnight without a significant reduction in the total urease activity or an increase in sample volume. Dialysing the sample against 25 mM-potassium phosphate buffer containing 100 mM-KCl did not prevent the Macro-Prep column from retaining the enzyme (Fig. 1). Washing the Macro-Prep column with RB2 containing 180 mM-KCl, then RB2 containing 220 mM-KCl, eluted > 90% of the total (non-urease) protein bound to the column. The application of an increasing KCl gradient (220 to 500 mM) eluted the enzyme in a single activity peak between 250 and 300 mM KCl.

Reducing the pooled Macro-Prep fractions from 3 ml to 500 µl with the Amicon concentrator did not result in a significant loss of enzyme activity. Concentrated sample was transferred to a S 300 column and eluted in a single enzyme peak. Fractions with the highest urease activity were pooled, and a small portion (200 µl) was prepared for SDS-PAGE. A single protein band of 83 kDa was visualized with Coomassie blue and silver stain on the SDS-PAGE from the pooled S 300 fractions (Fig. 2), thus the enzyme preparation appeared to be homo-
ducted with varying levels of urea (0 to 20 mM) and three hydroxyurea, and thiourea) can be inhibitory (Mobley & Hausinger, 1989). However, N-methylurea inhibits urease activity uncompetitively (Cornish-Bowden, 1979; Hammes, 1982) based on a Lineweaver–Burk plot. Uncompetitive inhibition of urease has been previously reported for halogens but so far, not for an analogue of urea (Mobley & Hausinger, 1989). In a review of microbial ureases, Mobley & Hausinger (1989) reported that N-methylurea acted as an inhibitor, but the mode of inhibition was unclear.

Estimation of native molecular mass. The native molecular mass of urease eluted from the S 300 column was estimated at 250,000, which is similar to previous findings (Christians et al., 1991; Creaser & Porter, 1985; Mobley & Hausinger, 1989). The single protein band of 83 kDa in the final enzyme preparation indicates that the protein could be composed of three identical subunits (Fig. 2). However, it is unclear if the 83 kDa band represents three monomeric subunits. Ureases are large (200 to 600 kDa) proteins composed of six or more subunits, varying from 16 to 60 kDa, and arranged in protein complexes of (αβ)₈ or (αβδ)₈ (Christians et al., 1991; Creaser & Porter, 1985; Dunn et al., 1991; Evans et al., 1991; Mobley & Hausinger, 1989; Rai, 1989). Although the enzyme preparation was homogenous, based on the SDS-PAGE protein bands visualized with silver stain, the subunit makeup of the enzyme is unclear.

Estimation of pH optimum. The purified enzyme demonstrated activity over a wide pH range (5 to 10) with an optimal pH of 8.0. The pH optimum of the purified urease was determined with citrate phosphate buffer (pH 3–7) and HEPPSO (pH 7–9). The range at which A. niger urease activity could be detected and estimation of optimal activity is similar to previous reports of urease activity (Christians et al., 1991; Creaser & Porter, 1985; Evans et al., 1991; Mobley & Hausinger, 1989; Rai, 1989; Zawada & Sutcliffe, 1981).

Acid ureases have been used to remove urea from wine (Famuyiwa & Ough, 1991; Fujinawa et al., 1990; Kakimoto et al., 1989; Ough & Trioli, 1988). When purified A. niger urease was immobilized on a Phenyl Sepharose column, the enzyme reduced urea concentrations in solutions containing ethanol levels (15% v/v) higher than wine (Table 2). However, decreasing the pH of the ethanol solution to 3-5 eliminated the urease's ability to metabolize urea. Therefore, the urease from A. niger would not be suited for the commercial scale removal of urea from wine. However, if the A. niger urease was immobilized on a matrix, this immobilized urease could be used to reduce the levels of urea in non-

![Image](image-url)
which is decreased by dialysis. If blood was circulated distress often have elevated levels of urea in their blood, low levels of Na,EDTA and DTT (Table 3). Na,EDTA while increasing levels of Na,EDTA (see above).

Urease activity was enhanced by, but not dependent on, substrate urea (70%)

Table 2. Activity of immobilized urease
Activity of urease (20 mg) purified from Aspergillus niger and immobilized on 10 ml Phenyl Sepharose. Two solutions (pH 4.0 and 7.0) containing 2 mM-urea and 15% (v/v) ethanol were circulated over the column at a flow rate of 10 ml min$^{-1}$ at 25 °C for 3 d. The pH of the solutions was maintained with 50 mM-sodium acetate (pH 4.0) and 50 mM-Tris/HCl (pH 7.0).

<table>
<thead>
<tr>
<th>Solution</th>
<th>Urea concn (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.0</td>
<td>2.0  2.0</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>2.0  0.3</td>
</tr>
</tbody>
</table>

Table 3. Influence of Na$_2$EDTA, DTT, cations, anions, acetate, citrate and nitrate on urease purified from Aspergillus niger
The activity of urease in the standard assay mixture was used as a control (100%). Cations were chloride salts, while anions, acetate, citrate and nitrate were sodium salts. Most values have less than 1% variability, based on the mean of six trials from two separate purified enzyme preparations.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Activity (% at</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM</td>
<td>5 mM</td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
<td>130.3  127.5</td>
</tr>
<tr>
<td>DTT</td>
<td>129.2  123.0</td>
</tr>
<tr>
<td>K$^+$</td>
<td>96.1    91.9</td>
</tr>
<tr>
<td>Li$^+$</td>
<td>88.6    86.5</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>101.2   98.9</td>
</tr>
<tr>
<td>Br$^-$_</td>
<td>126.2   129.4</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>116.7   117.1</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>15.4    15.0</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>79.5    70.7</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>94.1    92.4</td>
</tr>
<tr>
<td>Br$^-$_</td>
<td>100.5   101.8</td>
</tr>
<tr>
<td>Cl$^-$_</td>
<td>101.2   98.9</td>
</tr>
<tr>
<td>I$^-$_</td>
<td>66.5    43.4</td>
</tr>
<tr>
<td>Acetate</td>
<td>95.9    93.1</td>
</tr>
<tr>
<td>Citrate</td>
<td>120.6   135.8</td>
</tr>
<tr>
<td>Nitrate</td>
<td>102.3   101.0</td>
</tr>
</tbody>
</table>

Influence of Na$_2$EDTA and DTT on urease activity. Urease activity was enhanced by, but not dependent on, low levels of Na$_2$EDTA and DTT (Table 3). Na$_2$EDTA and DTT concentrations of $\leq$ 0.5 and 100 μM, respectively, enhanced urease activity by 31.2 and 30.1%, while increasing levels of Na$_2$EDTA (>$ 20$ mm) and DTT (> 5 mm) were inhibitory (data not shown). In the literature, the role of these two compounds is ambiguous, suggesting that the influence of Na$_2$EDTA and DTT is dependent on the specific urease analysed. Urease activity can be enhanced by EDTA, DTT and β-mercaptoethanol (Christians et al., 1991; Creaser & Porter, 1985; Evans et al., 1991; Mahadevan et al., 1977; Mobley & Hausinger, 1989; Rai & Singh, 1987). However, another possible explanation may lie in the purity of the enzyme extract. When initially optimizing the urease assay with a crude enzyme extract, all levels (> 1 to 20 mM) of Na$_2$EDTA and DTT were inhibitory (data not shown). This suggests that measurements of urease activity in crude enzyme preparations could be spurious because of interfering compounds or proteases affecting the apparent urease activity.

Influence of cations, anions, acetate, citrate and nitrate on urease activity. The urease assay mixture was supplemented with varying levels (0, 2, 5 and 20 mM) of cations (chloride salts) or anions, acetate, citrate and nitrate (sodium salts). Urease activity was not affected by Na$^+$, K$^+$, Cl$^-$, Br$^-$, acetate and nitrate, but decreased significantly with Li$^+$, Mg$^{2+}$, Ni$^{2+}$, Zn$^{2+}$ and I$^-$(Table 3). Surprisingly, urease activity was increased by a mean of 16.9, 27.8 and 28.2% by Ca$^{2+}$, Ba$^{2+}$ and citrate, respectively. The literature regarding the influence of metabolites on urease activity is highly contradictory (Mahadevan et al., 1977; Rai & Singh, 1987). For example, our results suggest that Ba$^{2+}$ enhances urease activity, while Mahadevan et al. (1977) found that Ba$^{2+}$ was inhibitory. However, general trends in the literature suggest that Na$^+$, K$^+$ and nitrates have no effect on urease activity, while Mg$^{2+}$, Ni$^{2+}$ and Zn$^{2+}$ are inhibitory (Table 3).

Thermostability and storage of urease. Purified urease was treated at 65, 80 and 100 °C through 30 min, then cooled to 4 °C and assayed for activity (Fig. 3). Urease activity decreased by 26.0% after 30 min at 65 °C, 86.5%
after 5 min at 80 °C and 100-0% after 1 min at 100 °C. These results are in agreement with previous studies of purified ureases. Ureases tend to be thermally stable up to 60 or 65 °C (Malthotra & Rani, 1969; Mobley & Hausinger, 1989).

The purified enzyme was transferred to 1.5 ml microcentrifuge tubes (100 µl per tube) and stored at 4, -20 and -80 °C. A 30-5% reduction in total activity was observed after 90 d at 4 °C. Freezing the samples at -20 and -80 °C for 2 h did not affect the enzyme activity. Storing the samples for 28 d at -20 and -80 °C reduced the activity by 20.5 and 22.2%, respectively. This data tends to agree with that of Mobley & Hausinger (1989), who demonstrated that storing ureases (without glycerol) at low temperatures can result in a significant loss of activity over time.

**Conclusions**

The data presented in this manuscript details the first isolation and characterization of urease from *A. niger*. The basic information (activation, inhibition, pH optimum and temperature stability) obtained from this study could be used in future applications of the enzyme. Further, the technology developed with the DEAE anion exchange column chromatography could be applied to the industrial purification of the enzyme. Our data indicates that when the purified *A. niger* urease was immobilized on a matrix, the immobilized enzyme reduced the levels of urea in non-acidic solutions (Table 2).

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**References**


