Biodegradation **12:** 317–323, 2001.
© 2002 *Kluwer Academic Publishers. Printed in the Netherlands.*

Isobutylidenediurea degradation by *Rhodococcus erythropolis*

Thomas Jahns* & Roswitha Schepp
Fachrichtung 8.3 Mikrobiologie, Universität des Saarlandes, Postfach 151150, D-66041 Saarbrücken, F.R.G.
(author for correspondence, e-mail: toja@mx.uni-saarland.de)*

Accepted 13 August 2001

Key words: fertilizer, isobutyraldehyde, isobutylidenediurea amidinohydrolase, *Rhodococcus erythropolis*, urea

Abstract

A new enzyme (isobutylidenediurea amidinohydrolase) catalyzing the hydrolysis of isobutylidenediurea (a condensation product of urea and isobutyraldehyde widely used as a slow-release nitrogeneous fertilizer) was characterized from a strain of *Rhodococcus erythropolis*. The enzyme was purified 1250-fold to apparent homogeneity and shown to hydrolyze the fertilizer to urea and isobutyraldehyde at a molar ratio of 2 : 1. No activity was observed with ureido- or other structurally related compounds. Its molecular mass was determined by native polyacrylamide gelelectrophoresis and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry to be 15 kDa (±2 kDa) and 16.4 kDa, respectively. Growth of the bacterium in the presence of isobutylidenediurea led to an increased expression of the constitutively synthetized enzyme.

Introduction

Several condensation products of urea and aldehydes are utilized as slow- or controlled-release nitrogen fertilizers; approximately 300,000 tons of urea-formaldehydes, urea-isobutyraldehydes and urea-crotonylaldehydes are produced annually for these purposes (Trenkel 1997). At variance with urea-formaldehyde condensation products, which are compounds of varying molecular mass (Alexander & Helm 1990), the result of the reaction of isobutyraldehyde with urea yields a single oligomer, isobutylidenediurea (IDU), consisting of two urea moieties linked via an aliphatic branched residue (Powell 1968). Approximately 100,000 tons of this product are annually utilized in Western Europe, Japan and the United States for fertilization purposes, mainly on greens and lawns. In Western Europe, IDU represents the largest group of the slow and controlled-release fertilizers totally utilized. Attempts have even been made to improve the nutritive value of urea for dairy cows by using IDU as a non-proteinaceous source of nitrogen for ruminants (Teller & Godeau 1986), and toxicological studies demonstrated the absence of developmental toxicity of IDU even at high concentrations (Hellwig et al. 1997).

Results from numerous field trials showed that environmental factors such as moisture, temperature and pH are essential causes affecting the hydrolysis and decomposition of urea-aldehyde condensation products (Trenkel 1997). Attempts to classify these fertilizers, i.a with regard to the mechanism of their degradation, led to their grouping into three patterns (Chiang 1970a): the microbial decomposition pattern (urea-formaldehydes), both the hydrolytic and microbial decomposition pattern (urea-crotonylaldehydes), and the abiotic hydrolytic pattern (IDU), even though an involvement of microbial activity in the degradation of IDU has been taken into consideration very early (Chiang 1970b).

Despite the fact that IDU is released as a fertilizer in high amounts into the environment, no detailed knowledge as to the mechanism of its possible microbial metabolism is available. In the present study, a strain of *Rhodococcus erythropolis* degrading this fertilizer was isolated from soil, and a new enzyme involved in the degradation of IDU was purified and characterized.

Materials and methods

Materials

All chemicals and reagents were of at least analytical grade. Casein-peptone soymeal-peptone (CASO) was from Merck, Darmstadt, Germany. High molecular weight standard proteins, jack bean urease and glutamate dehydrogenase were from Boehringer (Mannheim, Germany), the 10 kDa protein ladder was from Gibco BRL (Paisley, UK). Q-Sepharose FF, Phenyl- Sepharose HL, Sephacryl S300HR and MonoQ were purchased from Pharmacia (Freiburg, Germany). The media and the buffers used for enzyme purification were prepared with Milli-Q-purified water (Millipore, Bedford, Mass., USA). Isobutylidenediurea (IDU) was synthetized according to Powell (1968).

Organisms, growth conditions and preparation of extracts

A bacterium capable of utilizing IDU as a nitrogen source for growth was isolated by inoculating 1 g of garden soil in 200 ml of a mineral-medium described earlier (Schlegel et al. 1961), containing 0.2% (w/v) lactic acid as the carbon source and 0.1% (w/v) IDU as the sole source of fixed nitrogen. Cells were grown aerobically at 28 °C for 4 d, then 2 ml of the suspension were transferred to 200 ml of the same medium. After one more subculture in this medium, cells were diluted in 1% (w/v) NaCl and plated on solid media of the same composition, containing 1.8% (w/v) agar-agar. One fast growing colony was isolated after 48 h at 28 °C and used for the further experiments. Cells were grown to the early stationary growth phase in the mineral medium with 0.2 % (w/v) lactic acid as the carbon source and the nitrogen sources indicated in the experiments, harvested by centrifugation (10,000 × *g* for 20 min at 4 °C) and washed twice in 50 mM Na_2HPO_4, pH 7.5, 3 mM mercaptoethanol, 1 mM EDTA (buffer A). For the preparation of cell-free extracts, cells (about 0.2 g wet weight per ml buffer A) were disintegrated by ultrasonic treatment, using a Branson Sonifier B 12 with an output of 60 W for a total time of 30 s ml^{-1} at intervals of 10 s with cooling of the cell suspension in an ice bath. The supernatants obtained after centrifugation (30,000 × *g* at 4 °C for 60 min) of the broken cell suspensions were used as crude extracts in the enzyme assays and for the purification of the enzyme.

Enzyme and protein assays

If not indicated otherwise, all assays for isobutylidenediurea amidinohydrolase (IDU amidinohydrolase) activity were performed in 25 mM Na_2HPO_4, pH 8.0, 1.5 mM mercaptoethanol, 0.5 mM EDTA, with 5 mM IDU (only partially solubilized at this concentration) as a substrate, and purified enzyme or cell-free extract in appropriate dilutions. The amount of urea released from IDU was determined after between 30 min and 2 h incubation at 30 °C by the following method: the reaction was stopped by heating (100 °C, 5 min; this treatment did not result in degradation of IDU), cooled to 30 °C and the urea formed hydrolyzed by the addition of 2 μl of jack bean urease (200 U ml^{-1}) to 1 ml of the assay mixture. The resulting ammonium was then determined using the coupled enzyme assay according to Kaltwasser & Schlegel (1966). In the fractions eluted from the Phenyl-Sepharose HL column, which contained high concentrations of ammonium, IDU amidinohydrolase was determined by measuring the decrease of IDU concentration in the assay mixture using a colorimetric assay (Moore & Kauffman 1969). Urease activity was determined as described previously (Jahns 1995). Isobutyraldehyde was measured enzymatically according to Goedde & Langenbeck (1970). Enzyme activities are given in units (U), one unit corresponding to one nmol of substrate hydrolyzed per min. Protein was determined according to the Lowry method (Lowry et al. 1951).

Purification of IDU amidinohydrolase

All the steps in the purification of the enzyme were carried out at between 22 and 28 °C. Cell-free extract (30 ml) was loaded onto a Q-Sepharose FF column (2 cm^2 × 12 cm) connected to the LKB HPLC system, equilibrated with buffer A. Using a linear gradient from 0 to 1.0 M NaCl, the enzyme was eluted at a flow rate of 1.5 ml min^{-1} at between 0.25 and 0.30 M NaCl. The most active fractions from the ion exchange chromatography were pooled, adjusted to 1.0 M $(NH_4)_2SO_4$ by adding 3.0 M $(NH_4)_2SO_4$, centrifuged for 2 h at 45,000 × *g*, loaded onto a Phenyl-Sepharose HL column (2 cm^2 × 12 cm) connected to the LKB HPLC system and equilibrated with buffer A, containing 1 M $(NH_4)_2SO_4$. Using a linear gradient from 1.0 to 0 M $(NH_4)_2SO_4$, the enzyme was eluted at a flow rate of 1.5 ml min^{-1} at between 0.12 and 0.08 M $(NH_4)_2SO_4$. The three most active fractions from the hydrophobic interaction chromatography were pooled and applied to a Sephacryl S300HR column (4.9 cm^2

× 99 cm) connected to the LKB HPLC system. The elution buffer used was buffer A containing 0.1 M NaCl, with a flow of 0.45 ml min^{-1}. Fractions of 6 ml were collected and assayed for IDU amidinohydrolase activity. The three fractions containing the highest enzyme activity were pooled and loaded onto a Mono-Q HR5/5 column connected to the LKB HPLC system. The enzyme was eluted using a linear gradient from 0.1 to 1.0 M NaCl in buffer A at a flow rate of 1 ml min^{-1} at approximately 0.28 M NaCl.

Electrophoretic techniques

Seperation of denaturated proteins was carried out using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which was performed in a Biometra Minigel system (Biometra, Göttingen, Germany) according to Laemmli (1970). Native proteins in undenaturated samples were separated in the same system in the absence of SDS. The isoelectric point of desalted IDU amidinohydrolase was determined using Servalyte Precoates® with a pH-range of 3–10 (Serva, Heidelberg, Germany), using the LKB Multiphor apparatus (LKB, Bromma, Sweden). All gels were silver-stained as described by Merril et al. (1981).

MALDI-TOF mass spectrometry

One volume of desalted purified IDU amidinohydrolase (0.2 μg ml^{-1}) was mixed with one volume of saturated dihydroxybenzoic acid in 33% acetonitrile and 0.1% trifluoroacetic acid, and 1 μl of this mixture was placed on the center of a sample dish. Measurements were carried out with a REFLEX III laser desorption time of flight instrument (Bruker-Daltonik GmbH, Bremen, Germany) equipped with a nitrogen laser (λ = 337 nm, 3 ns pulse width). Positively charged ions were accelerated from the target in the pulsed mode to a final potential of 20 kV. All measurements were performed in the positive mode; therefore, the molecules were protonated, the apparent molecular mass (M) being $M + H^+$ (M + 1).

Reproducibility of results

The tables and figures show representative results from experiments, which were repeated at least twice. Other results are mean values of at least two independent experiments; the SD values did not exceed 20% of the mean.

Results and discussion

Description and growth of the strain

The release from nitrogen of isobutylidenediurea (IDU) is supposed to be controlled by low dissolution/solubility and abiotic hydrolysis (Waddington et al. 1977). When IDU was incubated in a sterile acidic aqueous solution at a pH-value of 5, a continuous release of urea was indeed observed (Figure 1); however, even at neutral or slightly alkaline pH, where abiotic decomposition by acid-catalyzed hydrolysis is absent or very slow (Figure 1), a rapid degradation of IDU was observed both in garden soil as well as in a buffered medium containing 2.5 mM IDU, inoculated with garden soil (results not shown). From such an enrichment culture at pH 8.0, a bacterium utilizing IDU as a sole source of nitrogen for growth was isolated and identified as a strain of *Rhodococcus erythropolis* by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). This species is known for its physiological versatility and capability to degrade a number of natural and anthropogenic compounds; utilizing IDU as a nitrogen source for growth, a doubling time of 4 h at the optimum growth temperature of 28 °C was measured. On mineral media containing 10 mM ammonium as a nitrogen source, a generation time of 2.5 h was determined. IDU was not utilized as a carbon source for growth in a mineral medium containing 0.2% (w/v) of this compound as the sole carbon source.

The formation of the enzyme occurred under any growth condition studied; in both complex (CASO) and mineral media, specific activities of approximately 6 U mg $protein^{-1}$ were measured in the absence of added IDU, regardless of the nitrogen source added (Table 1). Highest IDU degrading activities were observed in cells grown in the presence of IDU in mineral media, and high concomitant urease activities were measured under the growth conditions tested (Table 1). Cells grown in a mineral medium with ammonium as the nitrogen source and subsequently incubated in a medium containing IDU showed a rapid increase in IDU amidinohydrolase activity, accompagnied by an increase of extracellular ammonium released from this compound and a slow onset of cell growth (Figure 2); when IDU was depleted from the medium, the cells started to assimilate the ammonium released. On the other hand, the transfer of IDU-grown cells to a growth medium containing no IDU resulted in a slow decrease of enzyme activity concomitant with

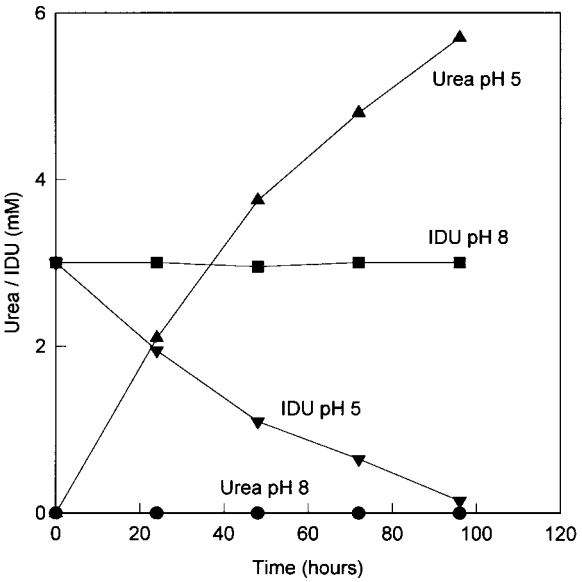

Figure 1. Abiotic hydrolysis of isobutylidenediurea at different pH-values. Filter-sterilized solutions of IDU in 20 mM phosphate pH 5 or pH 8 were incubated at 25 °C. At the times indicated, urea was determined by its hydrolysis to ammonia which was then measured using the coupled enzyme assay described in Methods, and IDU was determined colorimetrically according to Moore & Kauffman (1969).

Table 1. Specific IDU amidinohydrolase activity in *Rhodococcus erythropolis* grown with different nitrogen sources or incubated without nitrogen

Nitrogen source[1]	Specific activity [U (mg protein)$^{-1}$][2]	
	IDUase	Urease
Without nitrogen	6.2	123.5
2.5 mM IDU	30.2	766.5
10 mM NH_4Cl	6.1	85.0
10 mM NH_4Cl + 2.5 mM IDU	9.1	184.5
CASO	6.5	100.5
CASO + 2.5 mM IDU	6.3	128.2
10 mM KNO_3	5.9	111.2

[1] The cells were grown with 10 mM NH_4Cl to early exponential growth, harvested and resuspended in growth media containing 0.2% (w/v) lactic acid or 1% (w/v) CASO with the nitrogen sources indicated; they were then harvested during exponential growth, except the cells incubated without nitrogen, which were harvested after 24 h of incubation under nitrogen deficiency.

[2] One unit of activity equals the degradation of 1 nmol IDU (IDU amidinohydrolase, IDUase) or 1 nmol urea (urease) per min.

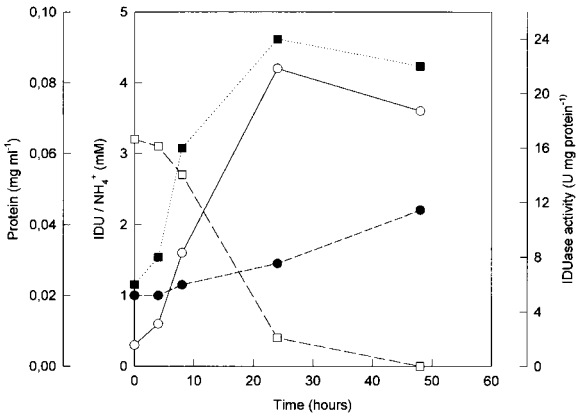

Figure 2. Growth of *Rhodococcus erythropolis* with isobutylidenediurea as the nitrogen source and IDU amidinohydrolase activity. Cells were grown with 20 mM ammonium as the nitrogen source to early exponential growth, washed once and resuspended in a mineral growth medium containing 2.5 mM IDU as the nitrogen source. □, IDU; ●, cell protein; ■, IDU amidinohydrolase (IDUase); ○, ammonium.

cell growth. These observations indicate an induction of the IDU amidinohydrolase by its substrate in the absence of other utilizable nitrogen sources, but also a constitutive synthesis even in the presence of other readily utilizable nitrogen sources. This may be of practical significance for the application of IDU as a slow-release fertilizer, since even in the presence of naturally occurring nitrogen sources, IDU degradation is not repressed.

We tested other *Rhodococcus* strains for their ability to metabolize IDU, and neither *R. erythropolis* strain DSM 43066 nor *Rhodococcus* sp. strain DSM 43001 hydrolyzed this compound when cells were pregrown in CASO and incubated for up to 72 h in media containing IDU as the sole source of fixed nitrogen for growth.

Purification of IDU amidinohydrolase, molecular mass and subunit composition

Using a procedure consisting of ion exchange chromatography, hydrophobic interaction and size exclusion chromatography, the enzyme was enriched 1250- fold from CASO-grown cells to apparent homogeneity and specific activities of about 7585 U × mg protein^{-1} with a yield of 36% (Table 2). Since the specific activities could not be determined exactly in the fractions eluted from the hydrophobic interaction column due to the high ammonium content in the eluent buffer, the purification parameters for this column were not determined (Table 2). It should be pointed out that

Table 2. Purification of the IDU amidinohydrolase from *Rhodococcus erythropolis*

Purification step	Total protein (mg)	Total activity (U)	Specific activity[1] [U (mg protein)$^{-1}$]	Yield (%)	Purification (fold)
Crude extract	123.5	749.6	6.07	100	1
Q-Sepharose	36.1	895.3	24.8	119	4
Phenyl-Sepharose	0.92	nd[2]	nd	nd	nd
Sephacryl S300HR	0.042	299.5	7131.0	40	1175
Mono-Q HR5/5	0.036	273.1	7584.8	36	1250

[1] One unit of activity equals the formation of 1 nmol urea from IDU per min.
[2] nd, not determined.

these activities were measured at suboptimal substrate concentrations, since IDU did not dissolve to more than 3.5 mM at the assay conditions used and the enzyme was not saturated at this IDU concentration (see below). Isoelectric focussing of the purified protein exhibited a single protein band at pH 3.8 to 4.0. Silver-stained gels from SDS-polyacrylamide gelelectrophoresis of the purified enzyme revealed one band corresponding to a molecular mass of 15 (±2) kDa (Figure 3, inset), and native PAGE [16 (±2)] as well as gel filtration [(16 (±5)] yielded a similar molecular mass for the undenaturated enzyme. A molecular mass of 16.4 kDa was determined by MALDI-TOF (Figure 3). These findings suggest a monomeric structure of the holoenzyme.

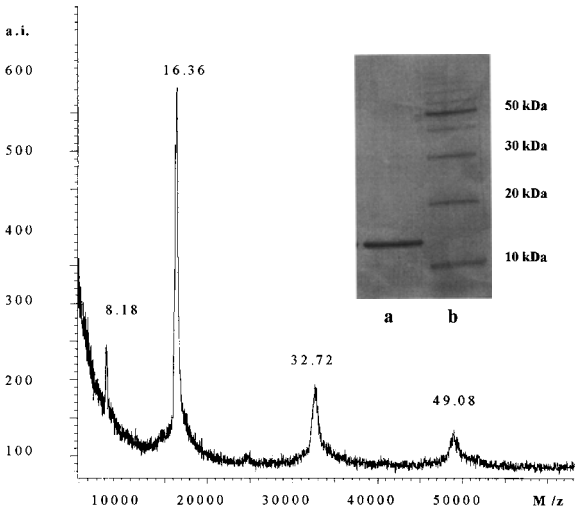

Figure 3. Molecular mass of the IDU amidinohydrolase, as determined by MALDI-TOF. M, mass; z, number of charges. a.i., absolute number of ions counted. The main peak (M/1z 16.36) corresponding to the mass of the purified enzyme + one charge ($1H^+$); a M/z of 8.18 corresponds to M/2z (+ $2H^+$); a M/z of 32.72 corresponds to 2M/z (+ $1H^+$); a M/z of 49.08 corresponds to 3M/z (+ $1H^+$). Inset: Molecular mass of purified IDU amidinohydrolase, as estimated by SDS-polyacrylamide gelelectrophoresis. Lane a, purified IDU amidinohydrolase (0.1 μg protein eluted at 0.28 to 0.30 mM NaCl from the Mono-Q HR5/5); lane b, low molecular weight standard (0.5 μl of 10 kDa protein ladder).

Kinetic properties, enzyme stability and substrate specificity

The purified enzyme was used for all subsequent studies. Optimum IDU amidinohydrolase activities were observed at pH 8.0 to 8.5 and 45 °C respectively; the enzyme was stable at between pH 4.5 and 11.0, loosing no activity upon incubation for 60 min at these pH-values; below pH 4.0 and above pH 11.3, a rapid irreversible loss of activity was observed (results not shown). The enzyme turned out to be unstable at elevated temperature, loosing 50% and 100% of its activity after 30 min at 52 °C and 60 °C, respectively. At low temperatures, the IDU amidinohydrolase was stable, since no loss of activity was observed when the enzyme was incubated on ice for up to 2 weeks or frozen at −70 °C in the presence of 20% glycerol for 4 weeks.

IDU was hydrolyzed to isobutyraldehyde and urea in a molar ratio of 2 : 1 (Figure 4), and the postulated degradation pathway of IDU is depicted in Figure 5. The enzyme was designated as an isobutylidenediurea amidinohydrolase; it acts on IDU by hydrolyzing this compound to urea and a postulated unstable intermediate, N-hydroxyisobutylurea (Figure 5, reaction A). Similarly to N-hydroxymethylurea (Bulygina et al. 1987), N-hydroxyisobutylurea is chemically unstable and decomposes abiotically to isobutyraldehyde and urea (Figure 5, reaction B). This reaction sequence differs from the degradation of a structurally related slow-release fertilizer, methylenediurea (MDU), which has been shown to be hydrolyzed to ammonium, carbon dioxide and an aminated methylurea by a methylene diurea amidohydrolase (Jahns et al. 1997). Attempts to determine the affinity (K_m-value) and the v_{max} of the IDU amidinohydrolase described in the present report failed, since even at the

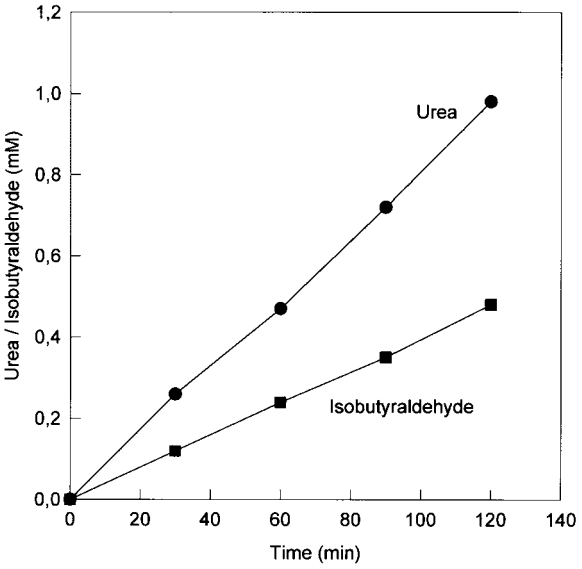

Figure 4. Formation of urea and isobutyraldehyde by purified IDU amidinohydrolase from Rhodococcus erythropolis. The purified enzyme was incubated at 30 °C in 25 mM Na_2HPO_2 in the presence of 2.5 mM IDU. At the time intervals indicated, urea and isobutyraldehyde were determined using the assays described in Methods.

highest IDU concentrations which could be tested (at pH 8.0 and 30 °C, a saturated aqueous solution contains approximately 3.5 mM IDU), the enzyme was not saturated. The purification of larger amounts of the IDU amidinohydrolase should permit its more detailed analysis.

Both MDU and IDU are structurally similar to allantoic acid, and interestingly, the degradation of allantoate (Vogel & van der Drift 1976, Xu et al. 1995) can occur both via an allantoate amidohydrolase (a reaction sequence analogous to the MDU amidohydrolase; Jahns et al. 1997, 1999) or an allantoate amidinohydrolase (a reaction sequence analogous to IDU amidinohydrolase described in the present report). While a low allantoate hydrolyzing activity was observed for the MDU amidohydrolase, the IDU amidinohydrolase did not hydrolyze this compound, and none of the following ureidylated or carbamoylated compounds served as a substrate for the enzyme: allantoin, urea, diureidomethane, N-methylurea, N-hydroxymethylurea, biuret, agmatine, ureidopropionate, D,L-citrulline, N-carbamoyl-α-amino-n-butyrate, ureidoacetate, N-carbamoylglycine, N-carbamoyl-L-glutamate, N-carbamoyl-L-leucine, N-carbamoyl-α-aminoisobutyrate, N-carbamoyl-D,L-aspartate, N-carbamoyl-DL-phenylalanine, N-carbamoyl-DL-alanine,

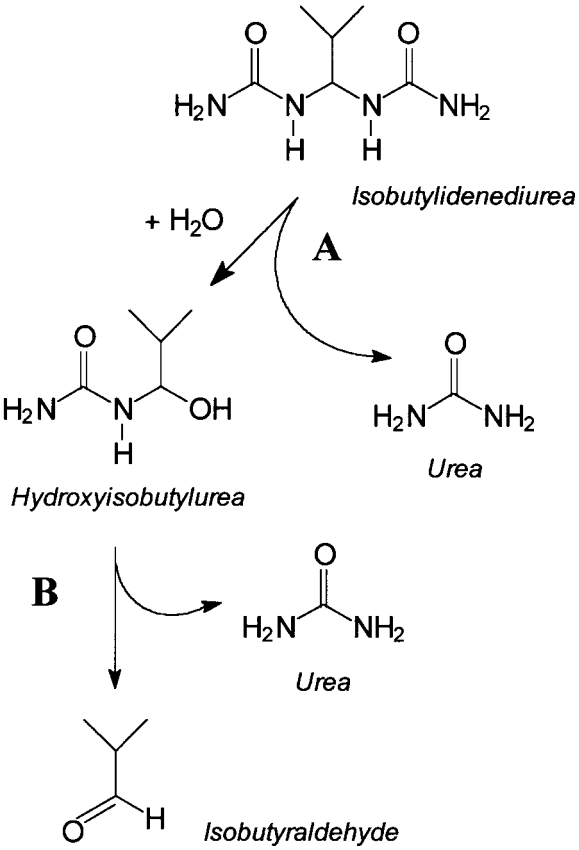

Figure 5. Proposed pathway of IDU degradation by *Rhodococcus erythropolis*. Reaction A, hydrolysis of IDU to urea and N-hydoxyisobutylurea catalyzed by the IDU amidinohydrolase; reaction B, abiotic decomposition of N-hydroxyisobutylurea to urea and isobutyraldehyde.

N-carbamoyl-DL-serine, N-carbamoyl-DL-norvaline, N-carbamoyl-DL-threonine and creatine.

Acknowledgements

The authors are indebted to K. Hollemeyer for the MALDI-TOF measurements. The financial support of this work by The Zentrale Forschungskommision der Universität des Saarlandes, Saarbrücken, Germany, is gratefully acknowledged.

References

Alexander A & Helm H-U (1990) Ureaform as a slow-release fertilizer: a review. Z. Pflanzenernähr. Bodenk. 153: 249–255

Bulygina SM, Gritsenko LP, Sokolovskaya IE & Sarbaev AN (1987) Hydroxymethyl derivatives of urea and formaldehyde: conditions of their formation. J. Appl. Chem. USSR 60: 419–421

Chiang CT (1970a) Decomposition and nitrogen transformation of slow-acting nitrogenous fertilizers in soils. Taiwan Fert. Co., Ltd., Res. Bull. 36: 1–45

Chiang CT (1970b) Decomposition and nitrogen transformation of slow-acting nitrogenous fertilizers in soils. Soils Fert. Taiwan 1970: 21–47

Goedde HW & Langenbeck U (1970) Glykolaldehyd. In: Bergmeyer HU (Ed), Methoden der enzymatischen Analyse (pp 1472–1474). Verlag Chemie Weinheim/Bergstr

Hellwig J, Klimisch HJ & Beth-Hubner M. (1997) Study on the developmental toxicity of orally administered isobutylidenediurea in rats. Food Chem. Toxicol. 35: 677–81

Jahns T (1995) Purification and properties of urease from *Sporobolomyces roseus*. Antonie van Leeuwenhoek 68: 209–214

Jahns T, Schepp R & Kaltwasser H (1997) Purification and characterization of an enzyme from a strain of *Ochrobactrum anthropi* that degrades condensation products of urea and formaldehyde (ureaform). Can. J. Microbiol. 43: 1111–1117

Jahns T, Schepp R, Siersdorfer C & Kaltwasser, H. (1999) Biodegradation of slow-release fertilizers (methyleneureas) in soil. J. Environm. Polymer Degrad. 7: 75–82

Kaltwasser H & Schlegel HG (1966) NADH-coupled enzyme assay for urease and other ammonia-producing systems. Anal. Biochem. 16: 132–138

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275

Merril CR, Goldman D, Sedman SA & Ebert MH (1981) Ultrasensitive stain for proteins in polyacrylamide gels show regional variation in cerebrospinal fluid proteins. Science 211: 1437–1438

Moore RB & Kauffman NJ (1969) Simultaneous determination of citrulline and urea using diacetylmonoxime. Anal. Biochem. 33: 263–272

Powell R (1968) Controlled release fertilizers. Chemical Process Review No. 15. Noyes Development Corporation, Park Ridge, New Jersey, USA

Schlegel HG, Kaltwasser H & Gottschalk G (1961) Ein Submersverfahren zur Kultur wasserstoffoxydierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. 38: 209–222

Teller E & Godeau JM (1986) Some attempts to improve the nutritive value of urea for dairy cows. II. Its administration in a slightly soluble form: isobutylidene diurea (IBDU). Arch. Tierernähr. 36: 419–28

Trenkel ME (1997) *Controlled-release and stabilized fertilizers in agriculture*. Ifa (International Fertilizer Association), Paris, France

Vogels GD & van der Drift C (1976) Degradation of purines and pyrimidines by microorganisms. Bacteriol. Rev. 40: 403–468

Waddington DV, Duich JM & Turner TR (1977) Turfgrass fertilization with isobutylidene diurea and ureaform. Proceed. Internat. Controlled Release Pestic. Symp. 1977: 319–334

Xu Z, de Windt FE & van der Drift C (1995) Purification and characterization of allantoate amidohydrolase from iBacillus fastidiosus. Arch. Biochem. Biophys. 324: 99–104