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An efficient enzymatic synthesis of 5-aminovaleric acid

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ABSTRACT

The title compound was prepared enzymatically from L-lysine in an excellent yield and under buffer-free conditions. L-Lysine was oxidized by the action of L-lysine α-oxidase from *Trichoderma viride* followed by spontaneous oxidative decarboxylation of the intermediate 6-amino-2-oxocaproic acid in the reaction medium. L-Lysine α-oxidase was immobilized on an epoxy-activated solid support (Sepabeads EC-EP) and the activity of both solution-based and immobilized enzyme in this reaction was determined.

1. Introduction

Polymeric substances are essential in modern society. With their applications in textiles, coatings, adhesives and plastics as well as their use in biomedical applications, synthetic polymers play an important role in our daily life. The building blocks for polymer production are highly dependent on fossil resources. Thus, hydrocarbon monomers like ethylene, propylene, and styrene are yielded from the refinement of crude oil, while nitrogen-containing building blocks like *e.g.* acrylamide, acrylonitrile and caprolactam are obtained by using ammonia (derivatives) at high temperatures and pressures in reactions with naphtha derived hydrocarbons, which is extremely energy-demanding [1,2]. Allied with this and other environmental concerns, there is a need to search for alternative sources of nitrogen-containing bulk chemicals. Plants are particularly attractive in this respect, as many products formed in plants (*e.g.* amino acids) already contain nitrogen.

In the course of our investigations of the use of plant materials as a feedstock for the bulk chemical industry [3], we have been involved in the application of enzymes for the conversion of amino acids into nitrogen-containing chemicals [4,5]. Enzymatic processes are environmentally friendly, and due to their intrinsic and selective catalytic properties, enzymes have found wide application in industrial synthesis of chemicals [6]. In the development of such environmentally benign production processes, lysine attracts attention as a suitable precursor in view of the availability of this amino acid from fermentation [7] or from lysine enriched plants. Thus, the accumulation of lysine in potato has been well investigated and described [8]. In the starting process of lysine transformations, L-lysine α-oxidase can be used.

L-Lysine α-oxidase (LysOx), a flavin-containing enzyme from the wheat fungus *Trichoderma viride*, catalyses the oxidation of the α-carbon atom of lysine (**1**, Scheme 1) [9]. For the oxidation of one mole of **1**, one mole of molecular oxygen is required. The formal product of this reaction is 6-amino-2-ketocaproic acid (**2**) [9], which is formed along with equimolar amounts of ammonia and hydrogen peroxide. If the reaction is conducted in the presence of catalase to remove H_2O_2, the final product represents Δ^1-piperideine-2-carboxylate (**3**, Scheme 1A) as a result of the spontaneous intramolecular cyclization of the intermediate. In the absence of catalase, the action of the enzymatically generated H_2O_2 on the intermediate leads to the oxidative decarboxylation of the latter to form 5-aminovaleric acid (**4**) as the final product (Scheme 1B).

Due to its high selectivity towards the oxidation of L-lysine, LysOx has attracted a great deal of attention, mainly as an instrument for the determination of L-lysine in various biological materials. A number of tools based on immobilized LysOx have been designed for this purpose [10–18], and the physicochemical and biological properties of this enzyme have been reviewed [19]. However, the reported applications of LysOx in synthesis are scarce and limited to its use as a part of the enzymatic systems for

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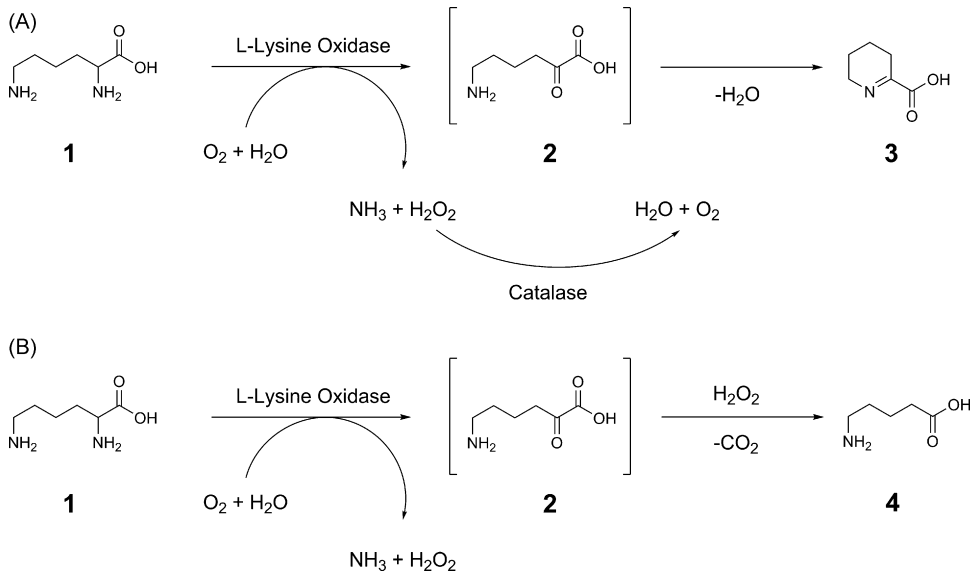

Scheme 1. Oxidation of lysine by L-lysine α-oxidase in the presence (A) and absence (B) of catalase.

the synthesis of L-pipecolic acid [20,21]. To the best of our knowledge, there is no report describing the isolation of either of the compounds **3** or **4** from the reaction mixtures followed by proper characterisation of these products, and to date, not much attention has been paid to the reaction in the absence of catalase (Scheme 1B). In our view, however, application of LysOx to the production of 5-aminovaleric acid (**4**) according to this scheme could be beneficial over the methods currently employed for the synthesis of **4** [22–28], as an environmentally friendly route that uses a biologically derived starting compound. 5-Aminovaleric acid may serve *inter alia* as the precursor of valerolactam, a potential building block for the production of nylon 5.

Thus, herein we describe the use of L-lysine α-oxidase (LysOx) on a preparative scale for the oxidation of lysine in the absence of catalase to produce 5-aminovaleric acid. An assay for LysOx for this reaction was developed and used in optimizing the reaction conditions. In addition, LysOx was immobilized onto an epoxy-activated support Sepabeads EC-EP and the performance of thus obtained enzyme preparation was compared to that of the soluble enzyme. Finally, we report on an efficient enzymatic synthesis of 5-aminovaleric acid with the use of the immobilized LysOx on a preparative scale and under buffer-free conditions, which allow for simplified product isolation.

2. Results and discussion

2.1. Development of an NMR-based assay for L-lysine α-oxidase

Focused on the route leading to the formation of 5-aminovaleric acid, we had to develop an assay for LysOx in this specific reaction, in view of inapplicability of the described assays hereto. Thus, in the reported procedures the enzyme is assayed by the reaction of the formal α-keto acid product (**2**, Scheme 1) with 3-methyl-2-benzothiazolone hydrazone hydrochloride or by determining Δ^1-piperideine-2-carboxylate with *o*-aminobenzaldehyde [9], as well as by reacting the oxidized product generated from lysine with semicarbazone [29]. However, in the absence of catalase the product of initial oxidation of the α-aminogroup of lysine does not lead to the compounds determined in conventional assay procedures. Therefore, for the quantification of product formation, a method that differs principally from those used in traditional endpoint and continuous LysOx assays has to be applied.

The use of ^{1}H NMR to determine the relative amounts of the starting lysine and the product in the reaction mixture is particularly attractive, given the simplicity of the ^{1}H NMR spectra of both compounds and only moderate overlapping of the multiplets belonging to **1** and **4**, especially at the lower pH values.

Moreover, contrary to the HPLC-based quantification of amino acids, neither additional functionalization, nor laborious sample preparation is needed prior to the measurement. In addition, the amounts of starting material used in preparative reactions do allow for ^{1}H NMR analysis of the reaction mixtures. Unfortunately, the necessity of oxygen for the enzyme functioning precludes from conducting the experiment outright in an NMR tube, so samples taken at the fixed time points of the reaction should be analyzed offline. It is important to stop the reaction immediately after sampling, in order to prevent any change in the composition of the reaction components prior (during) the NMR measurement. To this end, a solution of CF_3COOH can be used.

Conducting the reaction in D_2O allows avoiding the suppression of water ^{1}H signal that dominates NMR spectra of samples in H_2O and thus ensures more precise internal normalization. In this case, however, the formation of 5-amino-2,2-dideuterovaleric acid should be taken into account. In the spectrum of this compound, the multiplet at 2.35–2.25 ppm does not appear, and the integration of the signals in this region should not be included in the calculation of degree of conversion.

Based on these considerations, we performed the assay by determining relative concentrations of lysine and 5-aminovaleric acid from ^{1}H NMR spectra of the samples taken at given time points from the reaction mixtures containing initially 60 mM of lysine (Fig. 1).

The taken samples were immediately added to a 4% (v/v) solution of CF_3COOH in D_2O. The calculated percentages were plotted against time, and the activity of the LysOx was determined as a function of the slope of the resulting graph (Fig. 2).

Fig. 2 clearly shows, that the use of D_2O instead of H_2O in the reaction has no effect on the enzyme activity.

2.2. Optimization of the reaction conditions: effect of the nature of the buffer and temperature

In order to establish general principles for the reaction on a preparative scale, we first examined the oxidation of lysine by LysOx in a catalase-free reaction under conditions that are tradi-

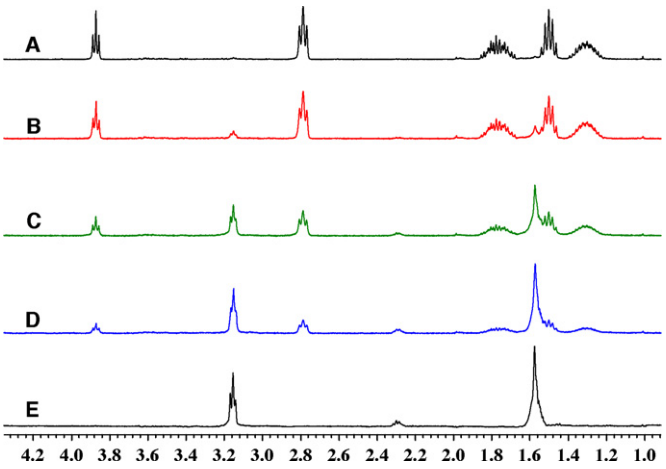

Fig. 1. NMR spectra of the reaction components for the conversion of lysine into 5-aminovaleric acid by L-lysine α-oxidase in D_2O. A—lysine, B–D—progress of the reaction (conversion of lysine 11% for B, 37% for C, and 69% for D), E—product.

tionally used for the assessment of the oxidative activity of this enzyme towards lysine in a reaction in the presence of catalase. Thus, a 70 mM phosphate buffer at pH 7.4 was used as a reaction medium and the reaction was conducted aerobically at 37 °C with initial lysine monohydrochloride concentration of 60 mM. The progress of the reaction was followed by NMR (*vide supra*) and the incubation was stopped when all the starting material was converted. The activity of LysOx in this reaction was found to be 8 times lower as compared to that determined in the standard assay for this enzyme in the presence of catalase. Whether this can be attributed to the detrimental action of H_2O_2 remains unclear, as all the formed hydrogen peroxide is consumed in the oxidative decarboxylation reaction according to the reaction scheme. It should be noted, that in spite of the observed activity difference, LysOx proved to be very stable at the applied conditions. Thus, even after 5 days of incubation, it showed oxidative activity towards lysine.

However, the isolation of 5-aminovaleric acid from the phosphate buffered reaction mixture proved to be challenging, prompting us to search for the alternative reaction media. In subsequent experiments, a solution of NH_4HCO_3 at pH 7.8 (containing 60 mM of lysine monohydrochloride), a 60 mM aqueous solution of the lysine free base, and a solution representing a mixture of lysine monohydrochloride and lysine free base at pH 7.4 and the total lysine concentration of 60 mM were used. Application of these

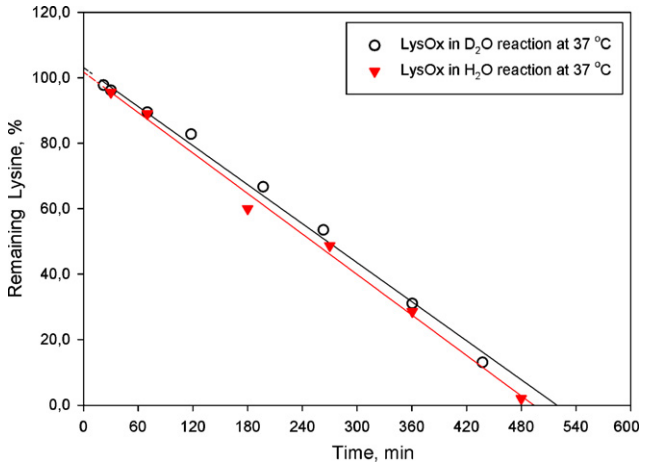

Fig. 2. Progress of the L-lysine α-oxidase-mediated oxidation of L-lysine in H_2O and D_2O.

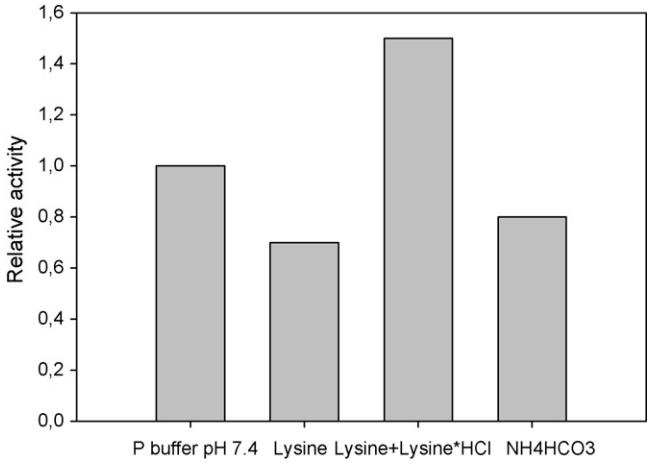

Fig. 3. Activities of L-lysine α-oxidase in different media.

solutions allows simplifying the product isolation, as an easily removable compound (NH_4HCO_3) or the substrate itself are used as the buffer basis. The activities of the LysOx in all the studied media were determined and compared (Fig. 3). From this comparison, the conclusion can be drawn that the aqueous solution of a mixture of lysine free base and lysine monohydrochloride at pH 7.4 is the buffer of choice for the reaction under study.

Increasing the temperature from 37 to 50 °C had little to no effect on the reaction rate, therefore in the subsequent experiments the reactions were conducted at 37 °C.

2.3. Immobilization of L-lysine-α-oxidase

In order to further facilitate the product isolation, we set out to catalyse the reaction by immobilized LysOx. Immobilization would also allow for an increased stability and reusability of the enzyme. Among the solid supports used for enzyme immobilization, the epoxy-activated ones have recently received an increasing attention owing to their ability to react with different functional groups on the enzyme surface to form extremely strong linkages with minimal chemical modification of the protein. In view of this and other properties, an epoxy-activated support Sepabeads EC-EP [4,30–32] was used in the present study.

Immobilization of LysOx on Sepabeads EC-EP was achieved by incubation of the enzyme solution in phosphate buffered saline with the solid support at room temperature for 36 h. High ionic strength was applied to ensure the hydrophobic adsorption of the protein onto the surface of the fairly hydrophobic Sepabeads. The adsorption is the first step in the two-step sequence of immobilization [31], and is followed by the covalent attachment of the adsorbed enzyme to the epoxy groups on the support surface.

After immobilization, the resulting enzyme preparation was filtered, washed subsequently with phosphate buffer and water (to ensure the desorption of any possible non-covalently attached protein) and the enzyme activity in the supernatant and washings was determined. The remaining enzyme activity in the supernatant was 2%, while the washings showed no activity at all. This allows assigning the immobilization yield at 98%.

2.4. An assay for the immobilized enzyme

The immobilized LysOx was assayed analogously to the enzyme in solution. Though stopping of the reaction in a taken sample was not necessary in this case, it still was inserted in the 4% (v/v) solution of CF_3COOH in D_2O prior to measurement, in view of a better resolution of the multiplets belonging to different reaction com-

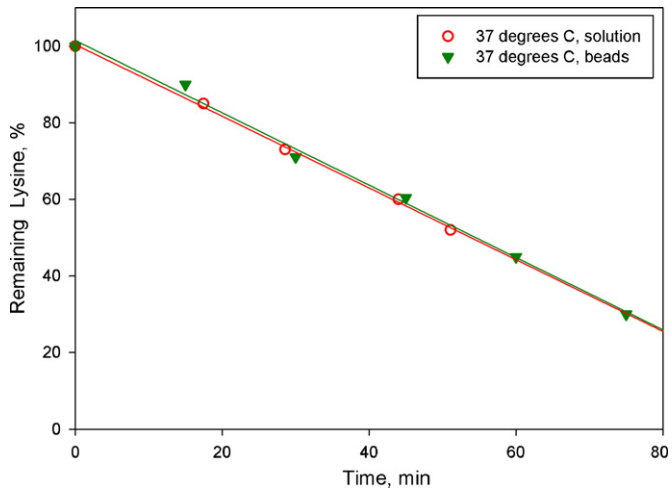

Fig. 4. Comparison of the activities of the immobilized and soluble L-lysine α-oxidase.

ponents at lower pH. Similarly to the soluble enzyme, for all the studied reaction media the immobilized LysOx showed maximum activity in the aqueous solution of a mixture of lysine free base and lysine monohydrochloride at pH 7.4.

Surprisingly, the activity of the immobilized enzyme was found to be at least equal to that of the enzyme in solution at the otherwise identical conditions (Fig. 4).

Apparently the immobilization provides a certain stabilization of the enzyme towards *inter alia* presence of H_2O_2 and the pH fluctuations induced by the constant formation of ammonia and CO_2 in the progress of the reaction without catalase.

However, the reusability of the immobilized enzyme was found to be only modest, as determined for the reaction at 37 °C in a mixture of lysine free base and lysine monohydrochloride at pH 7.4. Thus, when the immobilized enzyme was filtered off upon the complete conversion of the substrate, washed with phosphate buffered saline and water, and then used for the second time at the identical conditions, its activity in the second run consisted only 50% of that in the first cycle (data not shown). The supernatant obtained after filtration of the reaction mixture of the first run did not show any enzyme activity. This allows ruling out the possibility of the protein desorption during the reaction. Surprisingly, in the third cycle the activity of the immobilized enzyme did not drop further, and was equal to that in the second run (data not shown).

2.5. Synthesis of 5-aminovaleric acid

With the optimum conditions for enzyme functioning in hand, we performed a preparative reaction to synthesize 5-aminovaleric acid. Thus, the reaction mixture consisted of 50 ml of an aqueous solution of a mixture of lysine free base and lysine monohydrochloride at pH 7.4 with lysine concentration of 120 mM, and 3 U of immobilized LysOx. The mixture was incubated aerobically for 5 days at 37 °C after which NMR showed the complete conversion of the starting lysine. Filtering off the enzyme preparation and lyophilization of the reaction mixture allowed the target 5-aminovaleric acid in an excellent isolated yield of 95%.

3. Conclusions

We have studied the reaction of the lysine oxidation catalysed by L-lysine α-oxidase from *T. viride* in the absence of catalase. An NMR-based assay was developed and applied in the optimization of reaction conditions. The enzyme was successfully immobilized onto a solid support and the resulting enzyme preparation showed no loss of activity as compared to the enzyme in solution. The operational stability of the immobilized LysOx decreases after the first batch reaction but remains stable during further reactions. This indicates that a continuous process may be the best system for this enzyme. The immobilized enzyme was successfully used in the preparative synthesis of 5-aminovaleric acid.

4. Experimental

4.1. General information

L-Lysine α-oxidase from *T. viride*, L-lysine monohydrochloride and L-lysine monohydrate were purchased from Sigma. Sepabeads EC-EP were kindly donated by Dr. Paolo Caimi from Resindion S.R.L. (Mitsubishi Chemical, Milan, Italy). All other chemicals were obtained from Sigma or Fluka and were analytical or higher grade and were used as received.

NMR spectra were recorded on a 400 MHz Bruker Avance machine.

4.2. Solutions

A stock solution of L-lysine oxidase was prepared by dissolving the solid preparation obtained from Sigma in water to reach a protein concentration of 0.43 mg/ml. The lysine solutions used in this study were prepared as follows. Solution A: 0.06 M solution of lysine monohydrochloride in a 0.07 M phosphate buffer (pH 7.4 at 37 °C). Solution B: 0.06 M solution of lysine monohydrate in water. Solution C: 0.06 M solution of lysine monohydrochloride in a 0.1 M ammonium bicarbonate solution (pH 7.8 at 37 °C). Solution D: a 0.06 M solution of lysine monohydrochloride was titrated with a 0.06 M solution of lysine monohydrate to pH 7.4 at 37 °C. Solution E: a 0.06 M solution of lysine monohydrochloride in D_2O was titrated with a 0.06 M solution of lysine monohydrate in D_2O to pD 7.4 at 37 °C.

4.3. L-Lysine α-oxidase activity assay

Activity of the L-lysine oxidase was determined by measuring the change in concentrations of lysine and 5-aminovaleric acid in the course of the reaction. In a typical experiment, 2 ml of a 0.06 M solution of lysine (system A, B, C, D or E, *vide supra*) was pre-incubated at 37 °C for 5 min. The reaction was started by the addition of an appropriate aliquot of the stock solution of L-lysine oxidase and the whole was incubated at 37 °C (slow rotation of the mixture on a rotary evaporator) under a gentle flow of air. At given time intervals, 150 μl samples were taken followed by immediate inserting thereof into 300 μl of 4% (v/v) solution of CF_3COOH in D_2O to quench the reaction. Percentages of L-lysine and 5-aminovaleric acid were determined from a ^1H NMR spectrum of the resulting solution by comparison the sum of the integral values of the multiplets in 1.65–1.42 ppm region with the sum of the integral values of all the multiplets in the spectrum (when the reaction is performed in D_2O, the region of 2.35–2.25 ppm should not be integrated). The calculated percentages were plotted against time. The activity of LysOx was determined as a function of the slope of the resulting graph.

4.4. Immobilization of L-lysine α-oxidase on Sepabeads EC-EP

A total of 0.5 g of wet support was suspended in 2 ml of the enzyme solution (with a protein concentration of 0.025 mg/ml) in a 0.1 M phosphate buffer at pH 8 (the ionic strength of the buffer was adjusted to 5 M with NaCl). The mixture was incubated at 25 °C (slow rotation of the mixture on a rotary evaporator) for 36 h after

which the beads were filtered, and the activity of the supernatant was checked. The immobilized enzyme was washed by suspending it in 2 ml of a 0.01 M phosphate buffer at pH 8, followed by stirring at 25 °C for 10 min and filtering. This procedure was repeated twice, and finally the beads with the immobilized enzyme were washed on a glass filter with an excess of distilled water and stored as wet preparation at 4 °C.

4.5. Activity assay of the immobilized L-lysine oxidase

The activity of the immobilized LysOx was determined by quantifying the formation of 5-aminovaleric acid in the course of the reaction. A solution of lysine (B, D or E, 0.06 M, 2 ml) was pre-incubated at 37 °C for 5 min. The reaction was started by the addition of an appropriate amount of the immobilized enzyme and the whole was incubated at 37 °C (slow rotation of the mixture on a rotary evaporator) under a gentle flow of air. At given time intervals, 150 μl samples were taken followed by immediate insertion into 300 μl of 4% (v/v) solution of CF_3COOH in D_2O. Percentages of L-lysine and 5-aminovaleric acid were determined from a ^1H NMR spectrum of the resulting solution, and plotted against time. The activity of the immobilized LysOx was determined from the slope of the resulting graph.

4.6. Assessment of the operational stability of immobilized L-lysine α-oxidase

Two milliliters of a lysine solution (solution D, 0.06 M) were pre-incubated at 37 °C for 5 min after which the reaction was started by addition of 0.25 U of the immobilized enzyme. The whole was incubated at 37 °C (slow rotation of the mixture on a rotary evaporator) under a gentle flow of air. The reaction rate was determined by taking samples in time as described in the assay section. After 8.5 h of incubation NMR showed complete conversion of the starting material. The immobilized enzyme was filtered, washed with an excess of 0.07 M phosphate buffer pH 7.4 (or water) and added to a 2 ml solution of lysine (solution D, 0.06 M) that was pre-incubated at 37 °C for 5 min. The whole was incubated at 37 °C (slow rotation of the mixture on a rotary evaporator) under a gentle flow of air, and the rate of the reaction was determined as described in the assay section. The cycle was repeated for the third time, and activities of the immobilized enzyme in the first, second and third runs were compared.

4.7. Synthesis of 5-aminovaleric acid

The reaction mixture representing a suspension of the immobilized LysOx (3 U) in 50 ml of a lysine solution (solution D, 0.12 M, containing 0.88 g (6 mmol) of lysine) was incubated at 37 °C for 5 days by slow rotation on a rotary evaporation under a gentle flow of air. The progress of the reaction was followed by NMR. The enzyme was filtered off, and the supernatant was lyophilized to give 0.67 g (5.7 mmol, 95% yield) of 5-aminovaleric acid as a white solid.

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