APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY 6, 293–308 (1981)

Production of α-Keto Acids Part I. Immobilized Cells of *Trigonopsis variabilis* Containing D-Amino Acid Oxidase

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Received June 30, 1981; Accepted August 10, 1981

Abstract

Whole cells of *Trigonopsis variabilis* were immobilized by entrapment in Ca^{2+}–alginate and used for the production of α-keto acids from the corresponding D-amino acids. The D-amino acid oxidase within the immobilized cells has a broad substrate specificity. Hydrogen peroxide formed in the enzymatic reaction was efficiently hydrolyzed by manganese oxide co-immobilized with the cells. The amino acid oxidase activity was assayed with a new method based on reversed-phase HPLC. Oxygen requirements, bead size, concentration of cells in the beads, flow rate, and other factors were investigated in a "trickle-bed" reactor.

Index Entries: Production of α-keto acids; α-keto acids, production of; immobilized cells, in the production of α-keto acids; *Trigonopsis variabilis,* in the production of α-keto acids; D-amino acid oxidase, in α-keto acid production; oxidase, D-amino acid, in α-keto acid production; manganese oxide, co-immobilized with whole cells; liquid chromatography, assay of D-amino acid oxidase activity; chromatography, liquid, of D-amino acid oxidase activity.

Introduction

Patients suffering from acute uremia have a positive nitrogen balance and thus a poisonous excess of blood nitrogen that must be reduced as much as possible. This is done in an initial state of the disease, before dialysis is required, by administra-

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tion of a very restricted diet [low protein and high carbohydrate content *(1)*]. This diet is supplemented with the essential amino acids (including histidine for these patients).

If the essential carbon skeleton could be given in a "non-nitrogen" form, i.e, as the keto- or hydroxy-analogs of the amino acids, a more efficient treatment of this disease could be achieved, since these analogs would be transaminated in the body to remove some of the excessive nitrogen.

At present only five of the essential carbon skeletons are, however, commercially available in a "non-nitrogen" form (four keto acids, i.e., the analogs of valine, leucine, isoleucine, and phenylalanine, and one hydroxy acid, i.e., the analog of methionine) and, therefore, a more thorough investigation of this promising therapeutic approach has been hampered. The lack of suitable organic methods of synthesis of the remaining analogs has been a problem for several years and an alternative method for preparation is of great importance.

Many α-keto acids can be prepared from the corresponding amino acids by an enzymatic reaction involving oxidase according to Fig. 1. During recent years the use of immobilized biocatalysts for the preparation of pharmaceuticals has received increasing attention *(2)*. Such preparations have some distinct advantages and, therefore, the utilization of an immobilized amino acid oxidase for the production of α-keto acids appears to be suitable. Some studies have been carried out on immobilized amino acid oxidases by various investigators *(3–6)*. The enzyme utilized in these studies was isolated from hog kidney or snake venom. In a commercial process, however, an enzyme of microbial origin is preferred. We have found a suitable microbial source for D-amino acid oxidase in the yeast *Trigonopsis*

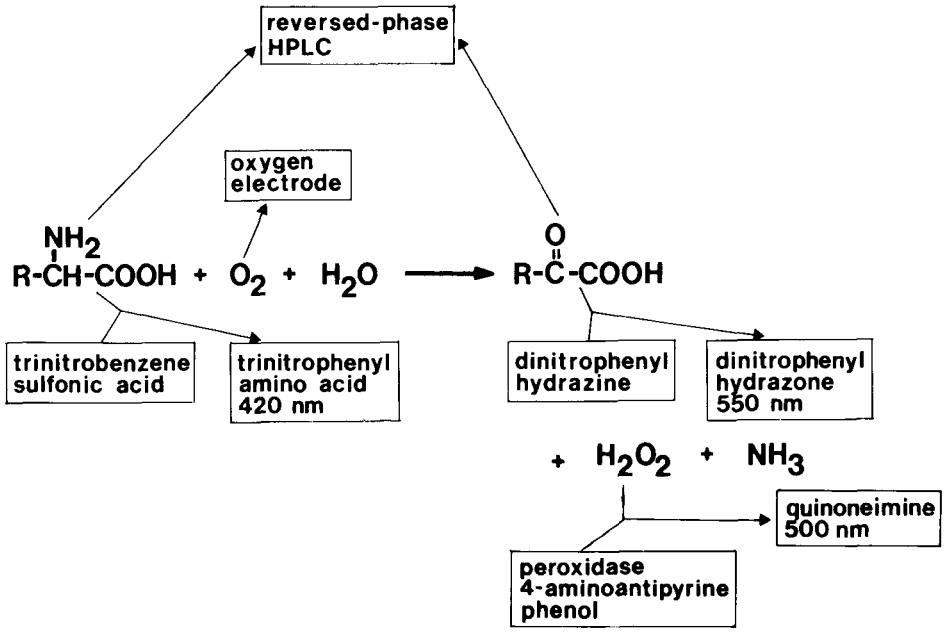

Fig. 1. Reaction scheme for the conversion of amino acids to the corresponding α-keto acids by amino acid oxidase and for various methods to assay the reactants.

variabilis. Whole cells of this microorganism were immobilized by entrapment in Ca^{2+}–alginate and used for the preparation of α-keto acids. A simultaneous production of L-amino acids is obtained when racemic mixtures of the substrates are utilized. In this paper the basic characteristics of the immobilized cells will be discussed and some process parameters investigated. A preliminary account of this work has been reported *(7)*. We are at present also studying immobilized L-amino acid oxidases of microbial origin and this work will be reported elsewhere *(8)*.

Materials and Methods

Chemicals

All amino acids and α-keto acids, as well as peroxidase (type II, from horseradish), alginic acid (sodium salt, type IV), dinitrophenyl hydrazine, and 6-amino-antipyrine were supplied by Sigma Chemical Co. Nucleosile-C_{18} (5 μm), methanol, manganese oxide, and calcium chloride were from Merck AG.

Cultivation of T. variabilis

Fermentation of the yeast *T. variabilis* was carried out essentially as described by Berg and Rodden *(9)*. The incubation was 10 days at room temperature in 5-L flasks with stirring and aeration. Cells were collected by centrifugation and then washed and stored frozen until used.

Immobilization of T. variabilis

Immobilization of *T. variabilis* cells was performed according to the procedure of Kierstan and Bucke *(10)* by entrapment in Ca^{2+}–alginate gels, according to the following standard procedures:

1. Wet cells (2.0 g) suspended in H_2O (3.0 mL) were mixed with 2% alginate (5.0 g) and subsequently the suspension was dripped into 0.1 *M* $CaCl_2$ (200 mL). After 2 h the beads were collected and washed with 50 m*M* Tris-HCl buffer, pH 8.0, containing 5m*M* $CaCl_2$.

2. Co-immobilization of cells and manganese oxide was achieved by adding the oxide (1.0 g) to the alginate solution (5.0 g) and this mixture was then sonicated to disintegrate larger aggregates of the oxide. The cells (2.0 g, suspended in H_2O, 2.0 mL) were added and the immobilization was carried out as described above.

Relatively large beads ($\phi \geqq 2.4$ mm) were obtained by pressing the mixture of alginate, cells, and maganese oxide through a syringe equipped with needles of various sizes. Smaller beads could, however, not be produced by this method owing to the high surface tension of the alginate suspension. Smaller beads ($\phi < 2.4$ mm) were made by mounting the syringe on a vortex mixer. By the oscillation of the needle, the surface tension of the viscous suspension was partly overcome, resulting in the formation of relatively small beads.

Assay Methods

The standard assay for determination of the D-amino acid oxidase activity of free and immobilized cells of *T. variabilis* applied in this investigation is a new method based on reversed-phase HPLC that permits simultaneous determination of substrate and product. In the case of free cells the samples were filtered (0.22 μm) before the analysis which was carried out in the following system:

Column size	200 × 4.6 mm
Column packing material	nucleoside-C_{18} (5 μm)
Mobile phase	methanol:water (2:8), pH 6.0
Flow rate	1.5 mL/min
Pressure	~1700 psi
Detection	UV, 210 nm

Standard curves were obtained by plotting the peak heights versus concentration of acid in standards. These standard curves were used for determination of amino and α-keto acids in the reaction mixtures.

The K_m and V_{max} values as well as the relative activity of the enzyme towards various substrates were, however, determined by two additional assay methods (see Fig. 1).

The activity of freely suspended cells was measured by forming the dinitrophenylhydrazone derivative of the keto acid produced. The sample (0.5 mL, containing 0–0.5 μmol α-keto acid) was mixed with saturated dinitrophenyl hydrazine in 2*M* HCl (0.2 mL). After 10 min, 3*M* NaOH (0.7 mL) was added and after an additional 15 min the absorbance at 550 nm was determined. Pyruvate was used as the standard.

The activity of immobilized cells was monitored by following H_2O_2 formation with a coupled enzymatic assay. The entrapped cells (0.2 g wet beads) were suspended by stirring in 50 m*M* Tris-HCl buffer, pH 8.0 (10 mL), containing 5 m*M* $CaCl_2$, 1 m*M* 4-amino-antipyrine, 10 m*M* phenol, peroxidase (approx. 10 U), and an appropriate concentration of substrate. The reaction mixture was continuously recirculated through a flow cuvette and the absorbance at 500 nm was recorded.

One unit of D-amino acid oxidase activity is defined as 1.0 μmol of α-keto acid formed per minute at 25°C and pH 8.0.

Results and Discussion

Enzyme Source

In previous studies on immobilized amino acid oxidases the source of the enzyme has been, from a commercial point of view, not very suitable. Enzymes isolated from hog kidney *(3)* or snake venom *(4, 5)* have been studied. The initial goal of this study was to find a good microbial source of the enzyme. From various possible microorganisms including bacteria, yeasts, and algae, the yeast *Trigonopsis variabilis* was selected as a suitable source. This yeast has previously been pointed

out as a source of D-amino acid oxidase *(9)*. Our choice was based on the following findings:

1. The enzyme is specific for the D-isomer of the substrate. Thus, optically active L-amino acids are produced simultaneously along with the keto acids from racemic mixtures of the substrate, which may increase the applicability of a process.

2. The enzyme is present in relatively large amounts in the yeast.

3. The enzyme shows relatively high activities towards most of the substrates of interest, i.e., the essential amino acids (see Table 1).

4. No side reactions are observed when whole cells are used for the oxidation, obviating the need for elaborate and expensive isolation of the enzyme.

Since intact cells of the yeast can be utilized without substantial formation of byproducts and since the purpose of this study was to develop a technical process, no attempts have been made to isolate and characterize the enzyme *per se*. The enzyme has, however, previously been partly purified *(9)*. In other applications of

TABLE 1
Relative Activity of D-Amino Acid Oxidase in Whole Cells of *T. variablis* Towards Various Amino Acids

Substrate	Relative activity, %
Methionine[a]	100
Ethionine[b]	78
Valine[a]	15
Norvaline[b]	55
Leucine[a]	40
Norleucine[b]	68
Isoleucine[b]	18
Phenylalanine[a]	91
Tyrosine[c]	nd
Tryptophan[a]	139
Histidine[a]	78
Lysine[b]	16
Arginine[b]	53
Citrulline[b]	74
Aspartic acid[b]	4
Aspargine[b]	32
Glutamic acid[b]	5
Serine[b]	7
Threonine[a]	2
Cephalosporin C[c]	nd

[a]10 m*M* D-amino acid as substrate.

[b]20 m*M* D,L-amino acid as substrate.

[c]Tyrosine and cephalosporin C are relatively good substrates, but because of their poor solubility, no relative activity has been determined. nd = not determined.

immobilized intact microbial cells problems with side reactions have occurred *(11, 12)*. A selective inactivation of the enzyme responsible for the side reaction has, however, solved these problems. Such inactivation has been achieved by heat treatment *(11)* or treatment with detergents *(12)*.

Enzyme Characteristics

As can be seen in Fig. 1, several methods are available for the determination of amino acid oxidase activity. Conventional assays for determination of substrate and products can be utilized, but the most convenient assay method is, however, a new one, developed by us, based on reversed-phase HPLC. With this method the amino and keto acid concentrations can be determined simultaneously. Furthermore, a measurement of the purity of the product is also obtained. The analysis is sensitive, accurate, and relatively rapid.

The broad substrate specificity is one of the major advantages of this enzyme and, as can be seen in Table 1, several amino acids can be converted to the corresponding α-keto acids with relatively high activity by intact cells of *T. variabilis*. Although the apparent K_m values for various substrates can vary considerably, from 0.5 to 2.0 m*M* for leucine, isoleucine, methionine, phenylalanine, tyrosine, tryptophan, and histidine up to about 20 m*M* for valine, the apparent V_{max} values for these substrates do not differ to such a great extent. Thus, some of the relative activities given in Table 1 have been determined at substrate concentrations below saturation and are, therefore, somewhat misleading. As can also be seen in Table 1, carboxy (aspartic and glutamic acid) and hydroxy (serine and threonine) amino acids are poor substrates. Methionine was selected as a representative substrate and it was used in the subsequent studies.

No significant difference in activity can be observed when D- or D,L-amino acids are used. The pure L-form of methionine is oxidized at a rate of approximately 2% of that observed with the pure D-form. Whether this results from the presence of small amounts of a L-amino acid oxidase or activity of the D-amino acid oxidase towards the L-isomer has not been investigated.

The pH optimum for the enzyme within the cells is 8.0 and the activity is increased with temperature up to about 50°C, at which temperature denaturation of the enzyme starts. The activity measurements were carried out within 15 min to avoid any significant influence by enzyme heat denaturation on the observed activities. At room temperature, where most of the experiments have been carried out, approximately 70% of the maximum activity is expressed. The enzyme appears to be relatively heat labile since treatment at 60°C for only 5 min results in complete loss of activity.

Immobilization of Whole Cells

For an industrial application of an immobilized biocatalyst a relatively simple method for immobilization should be employed. Furthermore, the polymeric matrix used must be relatively inexpensive. We feel that entrapment in Ca^{2+}–alginate fulfills these two fundamental requirements and, therefore, we have used this technique in these initial studies.

The substrate specificity as well as the apparent K_m and V_{max} values (determined for methionine, leucine, and phenylalanine) are not changed to any extent by the immobilization. Furthermore, the pH and temperature dependences are unaffected by the entrapment. Relatively large changes in these parameters can be observed upon immobilization of enzymes resulting from microenvironmental effects *(13, 14)*. In the case of whole cells, the enzyme is contained within a compartment and is, therefore, protected from changes in the microenvironment to some extent.

Hydrogen Peroxide

As can be seen in Fig. 1, hydrogen peroxide is formed in the enzymatic reaction. The requirement for the efficient degradation of this product is one of the main problems in developing a process for the production of keto acids. The removal of this product is important because of the following reasons:

1. Hydrogen peroxide is highly denaturating for proteins and, therefore, the operational stability of the immobilized biocatalyst will be influenced considerably by the presence of this compound.

2. A secondary reaction between hydrogen peroxide and α-keto acids can occur; i.e, the acid is decarboxylated, leading to decreased yield of product.

3. On the other hand, when hydrogen peroxide is degraded, oxygen is formed, which will favorably influence the reaction rate.

Various hydrogen peroxide degrading agents have been tested in similar systems, where peroxide is formed, e.g., glucose oxidase *(15)*. Co-immobilization of a second enzyme, catalase, with hydrogen peroxide degrading activity has been attempted *(15–17)*. The utilization of metal oxide-coated polymers as support for the immobilization of oxidases has shown promising results *(5)*. The metal oxides used have been those of manganese and ruthenium, which efficiently catalyze the degradation of hydrogen peroxide. The preparation of such coated polymers is, however, relatively complicated and, therefore, probably not of general applicability.

We have co-entrapped manganese oxide with the cells in the polymeric network of alginate in order to obtain an efficient degradation of the hydrogen peroxide formed. With this bifunctional catalyst the yield of product is close to quantitative (Fig. 2, solid symbols), while with a catalyst lacking the peroxide degrading capacity, on the other hand, the yield of product is decreased considerably owing to decarboxylation of the product (Fig. 2, open symbols). In Fig. 3, the HPLC elution profiles of samples from incubations with such catalysts are depicted. These profiles indicate that no by-products are formed when manganese oxide is co-entrapped with the cells (Fig. 3A) and that a byproduct (R_f = 2.3 min) actually can be seen when no manganese oxide is included in the catalyst (Fig. 3B). When hydrogen peroxide is added to a solution of α-keto-γ-methiol-butyric acid, a peak with R_f = 2.3 min, which increases in size with time, can be seen in the HPLC elution profile. The size of the α-keto acid peak is simultaneously decreased. The additional peak observed in the HPLC elution profile (Fig. 3B) is most likely the

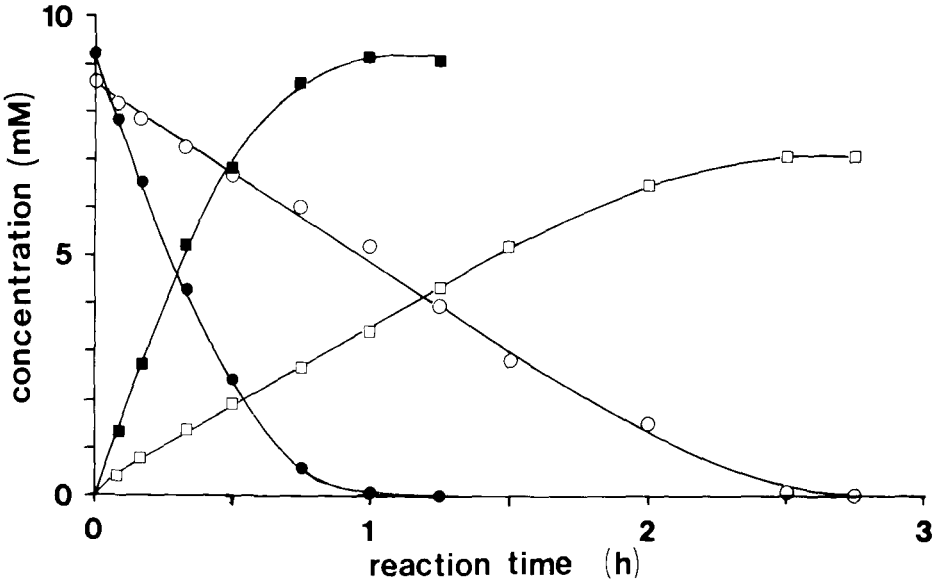

Fig. 2. Stoichiometry of D-amino acid oxidase reaction by immobilized cells of *T. variabilis*. A mixture of immobilized cells (0.2 g of wet beads, open symbols) or cells co-immobilized with manganese oxide (0.2 g of wet beads, solid symbols) and 50 m*M* Tris-HCl buffer, pH 8.0 (10 mL) containing 5 m*M* $CaCl_2$ and 10 m*M* D-methionine was incubated with stirring at room temperature. Samples were withdrawn and analyzed by HPLC. ●-●-● and ○-○-○ methionine, ■-■-■ and □-□-□ α-keto-γ-methiol-butyric acid.

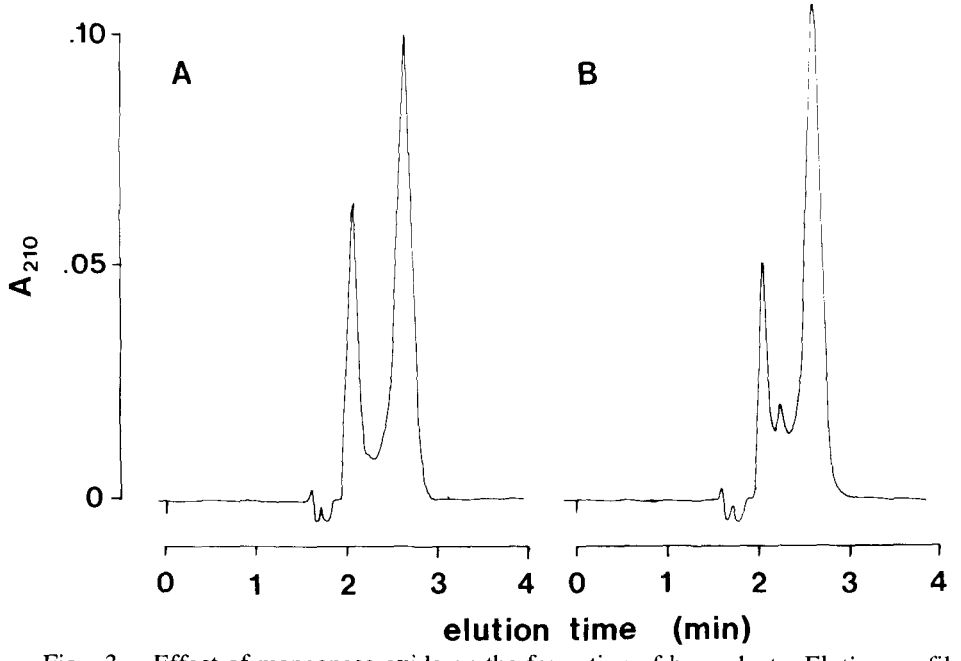

Fig. 3. Effect of manganese oxide on the formation of byproducts. Elution profiles from HPLC of samples from incubations of co-immobilized cells of *T. variabilis* with manganese oxide (A) or immobilized cells only (B). The incubations were carried out as described in the legend to Fig. 2, with additional bubbling of air through the mixtures (360 mL/min). The samples analyzed were taken after 60 min.

result of a secondary reaction between the keto acid and hydrogen peroxide, i.e., decarboxylation of the former as outlined above, and the byproduct formed is presumably β-methiol-propionic acid.

It can also be seen in Fig. 2 that the presence of manganese oxide considerably increases the reaction rate that probably results from recycling of oxygen since, if such incubations are carried out under very good aeration conditions, no significant difference in reaction rate is observed (cf. Fig. 3). It should be pointed out that at higher productivities the amount of byproduct, i.e., decarboxylated product, is increased considerably unless manganese oxide is co-entrapped with the cells. Furthermore, the preparation of the catalyst with hydrogen peroxide degrading capacity is very simple.

Finally, it can be seen in Fig. 4 that the enzyme activity is considerably stabilized by the manganese oxide. With no degradation of the peroxide, the half-life of the biocatalyst is only about 10 h. The denaturation of the enzyme by the hydrogen peroxide formed is, however, efficiently prevented by the metal oxide present in the beads.

Process Parameters

In the following we would like to discuss some parameters related to the design of a process for the large-scale production of α-keto acids by immobilized amino acid oxidase, which presents some unique problems. The requirements for high oxygen concentration and low hydrogen peroxide concentration in the reactor are the most important problems that have to be solved. In addition to this, the stability of the

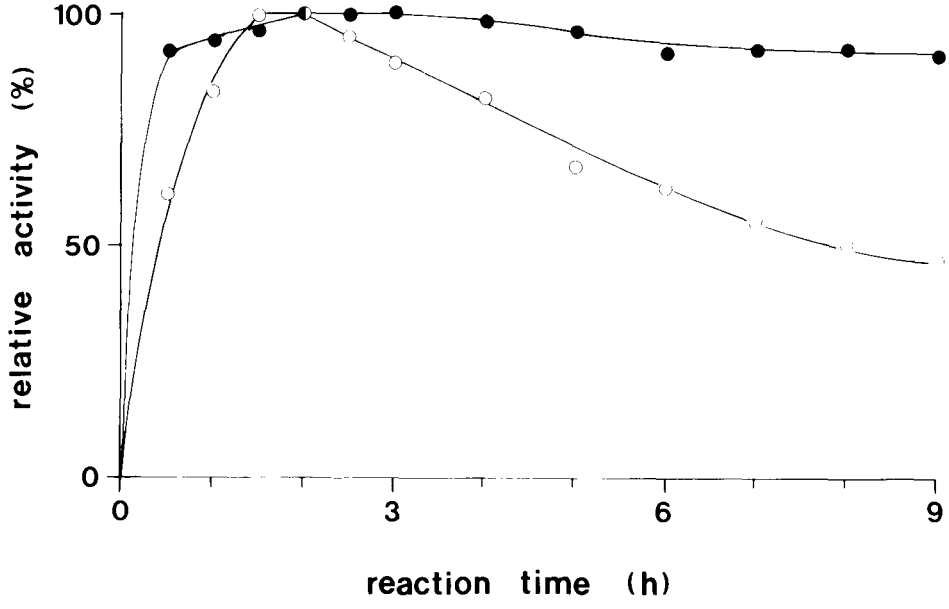

Fig. 4. Effect of manganese oxide on the operational stability of D-amino acid oxidase within immobilized cells of *T. variabilis*. A continuous incubation of immobilized cells (○-○-○) or co-immobilized cells with manganese oxide (●-●-●) was carried out as described in the legend to Fig. 3, with the additional replacement of substrate solution at a flow rate of 10 mL/h.

enzyme within the immobilized cells is a critical factor in the development of a technical process.

Since oxygen is one of the reactants in the oxidation of amino acids to the corresponding keto acids, it is necessary to design the reactor in such a way that an efficient oxygen transfer from the gas phase (air) to the enzymatic catalyst within the beads is obtained. To achieve this a number of different reactor designs are possible, such as a continuously stirred tank reactor, a packed-bed reactor, or a fluidized-bed reactor.

In a packed-bed reactor with parallel-current flow of air and the liquid phase, a so called "trickle-bed" reactor, problems with flooding can be avoided. Problems of this kind can easily occur in a packed-bed reactor with countercurrent flow of the substrate solution and air. In the "trickle-bed" reactor the liquid phase flows down the column as a thin film over the beads leading to a minimized diffusion barrier for oxygen from the air to the catalyst. We have used this type of reactor for further studies of some operational parameters of a process for the production of keto acids.

In principle other reactor types can be used. In a stirred-tank reactor, which is relatively simple to aerate, one of the main problems is, however, the limited me-

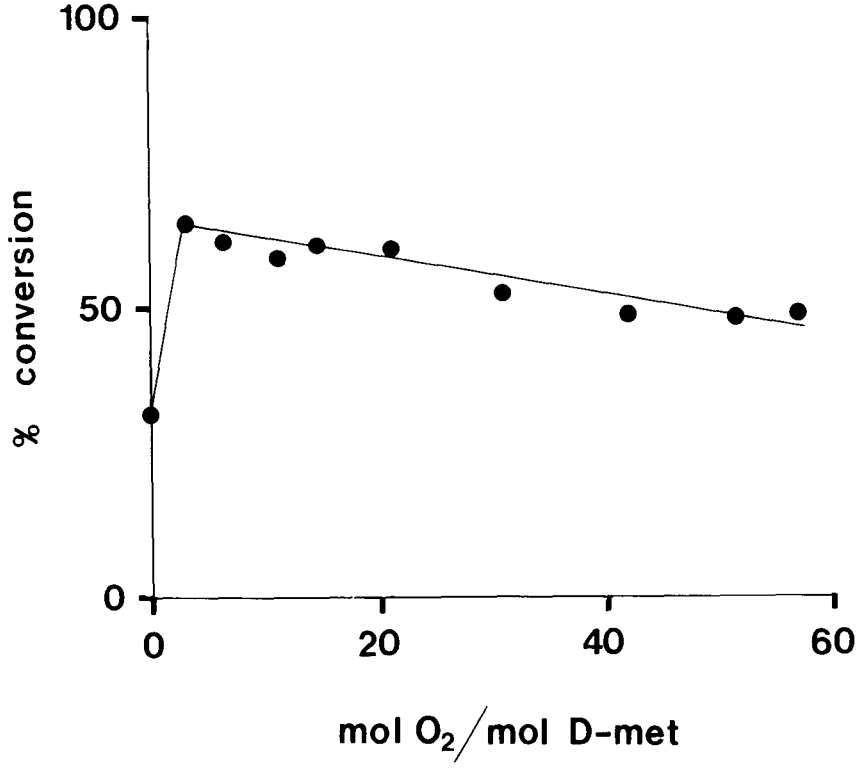

Fig. 5. Effect of oxygen to D-methionine molar ratio on the conversion of D,L-methionine to α-keto-γ-methiol-butyric acid. The beads (ϕ, 1.6 mm, 5.0 g wet weight) were packed in the reactor (16 × 100 mm) and the substrate (20 m*M*, D,L-methionine) was pumped through the reactor at a constant flow rate (28.8 mL/h) at various air flow rates until steady state was reached.

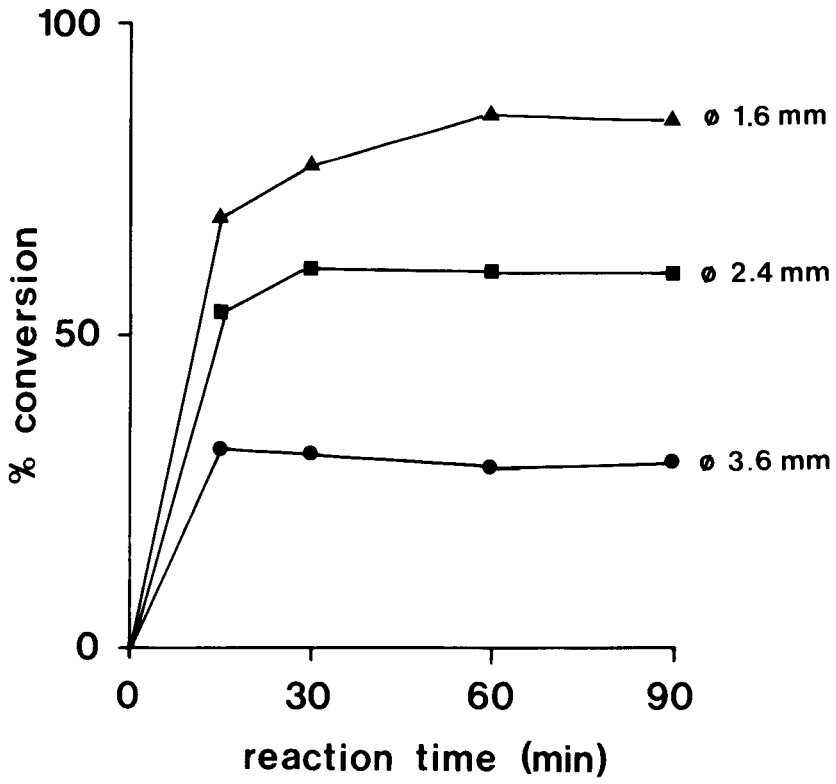

Fig. 6A. Effect of bead size on the conversion of D-methionine to α-keto-γ-methiolbutyric acid. The beads (5.0 g wet weight) were packed in the reactor (16 × 100 mm) and the substrate (20 m*M*, D,L-methionine) was pumped through the reactor (16.0 mL/h) and air was passed through the reactor (20 mL/min).

chanical stability of the alginate beads. A fluidized-bed reactor may also be employed.

By varying the flow rate of air through the column at a constant substrate flow rate, the requirement for oxygen for maximum productivity can be estimated. As can be seen in Fig. 5, maximum conversion is reached at an oxygen-to-substrate molar ratio of 5 under the conditions used. At higher oxygen concentrations, i.e., higher air flow rates, a slight decline in the conversion is observed. This decreased conversion can be explained by the accelerated flow rate of the liquid phase arising from the relatively high flow rate of the gaseous phase and/or by the occurrence of small droplets of substrate in the airstream. In a "trickle-bed" reactor the flow of air should thus be carefully adjusted for optimum productivity.

An increase in the effective transfer area between the gaseous phase and the catalyst, obtained by decreasing the size of the beads, results in an increased conversion, as shown in Fig. 6A. Furthermore, an increase in the steady state productivity is observed with increasing surface area within the reactor, as indicated in Fig. 6B. This is somewhat surprising, but it can be explained by assuming that a very diffusion-limited conversion is obtained under the conditions used. In such a case only the cells in the outer layer of the beads are effective in the catalysis and the productivity will be proportional to the effective surface area. An oxygen transfer

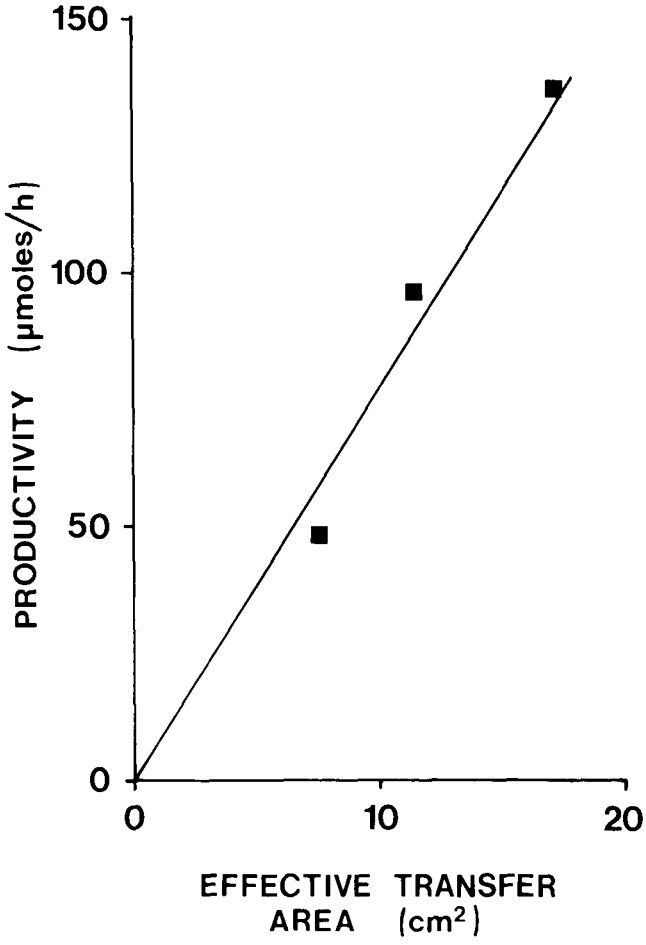

Fig. 6B. Steady-state productivity as a function of effective transfer area in the reactor. Calculations based on the data shown in Fig. 6A.

rate of 3–4 μmol $O_2/cm^2 \cdot h$ can be calculated for these three different reactors. This increased productivity points out the importance of having beads that are as small as possible, but at the same time, the risk for channeling within the reactor rises as the bead size decreases. In addition to this, very small beads might give rise to increased pressure drop over the column, which may cause problems with the flow rate. Therefore, a compromise among bead size, flow properties, and productivity must be found.

As can be seen in Fig. 7, the conversion (productivity) is highly dependent on the cell content of the beads at low cell densities ($< 10\%$). At higher cell densities, on the other hand, the productivity appears to be almost independent of the cell content of the beads. Obviously, the conversion is highly diffusion-restricted at these high cell densities. These diffusion limitations can to some extent be overcome by increasing the flow rate. As can be calculated from Fig. 7, the productivity is increased from 27.4 to 38.6 μmol/g beads/h when the flow rate is doubled (from 13.7 to 24.4 mL/h). Furthermore, the productivity with two volumes of beads containing 20% cells (77.2 μmol/g beads/h) is 170% of that obtained with

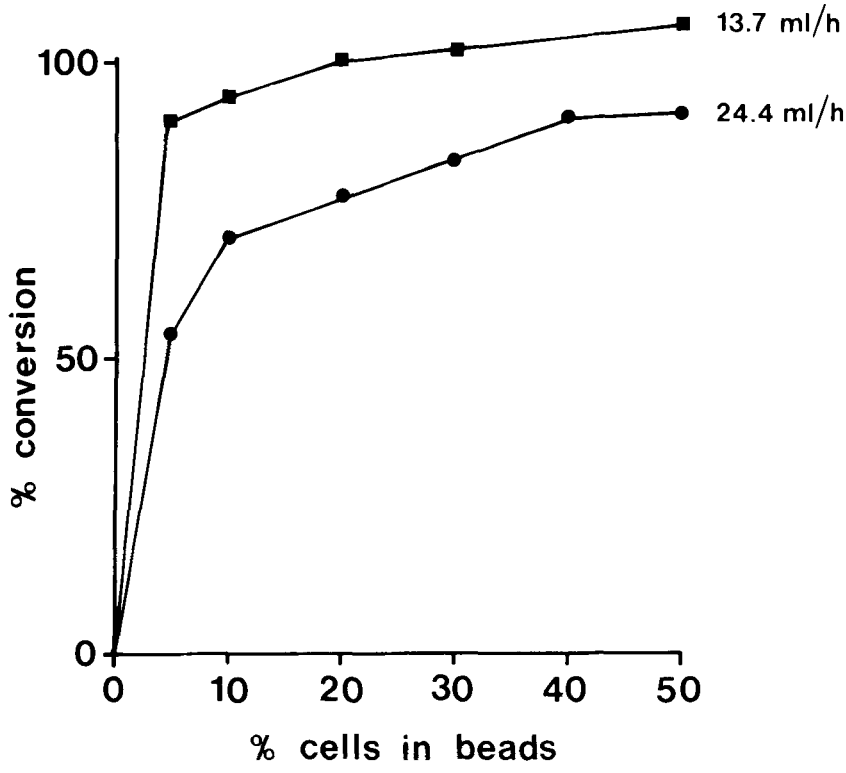

Fig. 7. Effect of cell content of the beads on the conversion of D-methionine to α-keto-γ-methiol-butyric acid. The beads (ϕ, 1.6 mm, 5.0 g wet weight) were packed in the reactor (16 × 100 mm) and the substrate (20 m*M*, D,L-methionine) was pumped through at the flow rates indicated with air passing the reactor (20 mL/min).

one volume of beads containing 40% cells (44.9 μmol/g beads /h). Thus, a compromise between cell content of the beads and reactor volume also has to be made for optimum productivity.

The conversion shown in Fig. 8 is also highly dependent on the flow rate of substrate through the reactor. Depending on the aim of the process, i.e., simultaneous production of keto acid and L-amino acid or production of keto acid alone, different flow rates should be applied. In the former case a complete conversion of the D-isomer is required in order to simplify the separation of the two products that can be achieved by extraction or ion exchange chromatography. In the latter case the flow rate should be set at as high a rate as possible for maximum productivity. At a high productivity the conversion is, however, relatively low and, therefore, an efficient recycling system for the amino acid is required.

From the relatively limited data of these model studies it is possible to make some extrapolations. With a reactor of 200 L volume a monthly production of either 1300 kg of keto acid (calculated at 40% conversion) or 540 kg of keto acid and 470 kg of L-amino acid could be achieved assuming constant activity of the biocatalyst over this period of time.

The operational stability observed for the D-amino acid oxidase within the immobilized cells of *T. variabilis* (about 7 days), is, however, not fully satisfactory

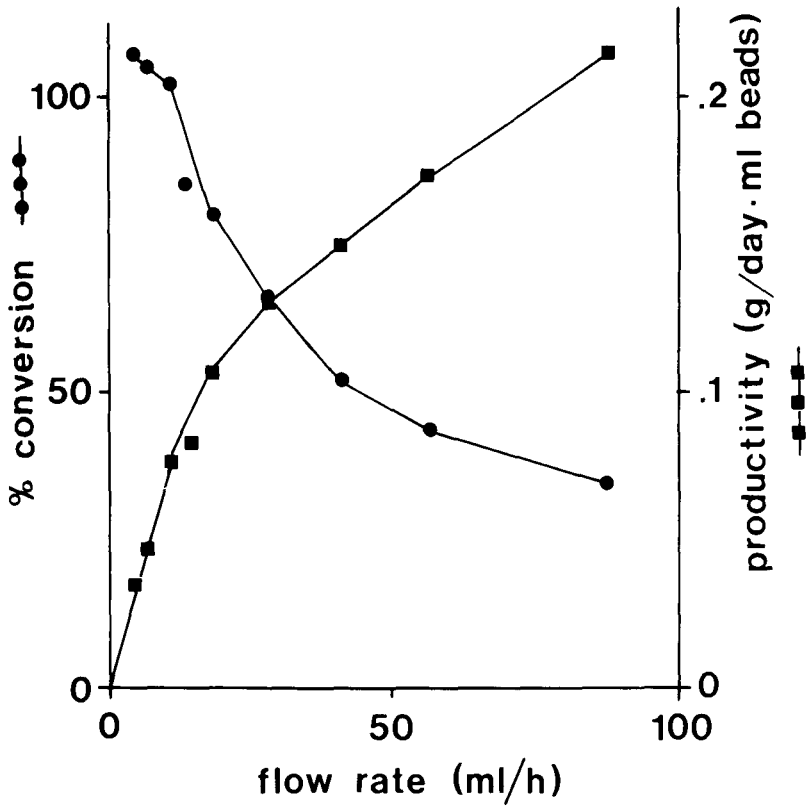

Fig. 8. Effect of flow rate of substrate on the conversion of D-methionine to α-keto-γ-methiol-butyric acid. The beads (ϕ, 1.6 mm, 1.5 g wet weight) were packed in the reactor (16 × 100 mm) and the substrate (20 m*M*, D,L-methionine) was pumped through the reactor at various flow rates with air passing the reactor (2.8 mL/min).

for a large-scale operation. This is in any event a considerable improvement compared to previous attempts to produce keto acids with immobilized amino acid oxidases where a half-life of only 2 h was found with D-amino acid oxidase from hog kidney immobilized on porous glass *(4)*. The half-life could be increased to 32 h by including FAD in the substrate stream. Furthermore, only low substrate concentrations were tested (<1 mM), but still only a small part was converted to the corresponding keto acid. We are now attempting to stabilize the enzyme within the immobilized cells in order to increase the operational stability.

Conclusions

The data presented here show that immobilized whole cells of *T. variabilis* may be utilized for the production of α-keto acids. Some of the advantages of the system are the following.

1. A microbial source for the enzyme.
2. A relatively broad substrate specificity of the enzyme.
3. Whole cells can be used without side reactions. No need for elaborate and expensive isolation of the enzyme.

4. Inexpensive and stable hydrogen peroxide-degrading agent can be used.

5. Complete conversion of D-amino acids is possible even at high substrate concentrations, which makes it feasible to produce optically active L-amino acids simultaneously with the α-keto acids.

6. Air can be used as oxygen supply.

Acknowledgments

The authors express their gratitude to Dr. S. Ohlsson for valuable discussions concerning the HPLC analysis. This work was supported in part by a grant from the National Swedish Board for Technical Development.

References

1. Bergström, J., Fürst, P., Josephson, B., and Norée, L.-O. (1972), *Nutr. Metabol.* **14,** Suppl. 162–170.
2. Brodelius, P. (1978), in *Advances in Biochemical Engineering,* vol. 10, Ghose, T. K., Fiechter, A., and Blakebrough, N., eds., Springer-Verlag, New York, pp. 75–129.
3. Tosa, T., Sano, R., and Chibata, I. (1974), *Agr. Biol. Chem.* **38,** 1529.
4. Fink, D. J., Falb, R. D., and Bean, M. K. (1976), in *Advances in Enzyme Engineering,* vol. 2, Tsao, G. T., ed., Purdue University, West Lafayette, Indiana, pp. 79–113.
5. Fink, D. J., and Allen, B. R. (1977), presented at the 3rd US/USSR Conference on "Development of Methods for Production of Enzymes and Their Application for Industrial and Analytical Purposes," Tallin, Estonia, USSR, November 28–December 2, 1977.
6. Naoi, M., Naoi, M., and Yagi, K. (1978), *Biochim. Biophys. Acta 523,* 12.
7. Brodelius, P., Hägerdal, B., and Mosbach, K. (1980), in *Enzyme Engineering,* vol. 5, Weetall, H. H., and Royer, G. P., eds., Plenum Press, New York, pp. 383–387.
8. Szwajcer, E., Brodelius, P., and Mosbach, K., to be published.
9. Berg, C. P., and Rodden, F. A. (1976), *Anal. Biochem.* **71,** 214.
10. Kierstan, M., and Bucke, C. (1977), *Biotech. Bioeng.* **19,** 387.
11. Yamamoto, K., Sato, T., Tosa, T., and Chibata, I. (1974), *Biotech. Bioeng.* **16,** 1601.
12. Yamamoto, K., Tosa, T., Yamashita, K., and Chibata, I. (1976), *Eur. J. Appl. Microbiol.* **3,** 169.
13. Katchalski, E., Silman, I., and Goldman, R. (1971), in *Advances in Enzymology,* vol. 34, Nord, F. F., ed., Wiley, New York, pp. 445–536.
14. Goldstein, L. (1976), in *Methods in Enzymology,* vol. 44, Mosbach, K., ed., Academic Press, New York, pp. 397–443.
15. Messing, R. A. (1974), in *Immobilized Enzymes in Food and Microbial Processes,* Olson, A. C., and Cooney, C. L., eds., Plenum, New York, pp. 149–156.
16. Bouin, J. C., Atallah, M. T., and Hultin, H. O. (1976), in *Methods in Enzymology,* vol. 44, Mosbach, K., ed., Academic Press, New York, pp. 478–488.
17. Danielsson, B., Gadd, K., Mattiasson, B., and Mosbach, K. (1977), *Clin. Chim. Acta* **81,** 163.