COMMONWEALTH OF AUSTRALIA

Patents Act 1952-1969

CONVENTION APPLICATION FOR A PATENT

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We,

HOECHST AKTIENGESELLSCHAFT,

of 45 Bruningstrasse, D-6230 Frankfurt/Main 80,

Federal Republic of Germany

hereby apply for the grant of a Patent for an invention entitled:

L-AMINOACID OXIDASE FROM YEASTS OF THE GENUS
CRYPTOCOCCUS, THEIR PREPARATION AND USE

which is described in the accompanying complete specification. This application is a Convention application and is based on the application numbered P33 33 453.6

for a patent or similar protection made in Federal Republic of Germany on 16th September 1983.

Address for service is Messrs. Edwd. Waters & Sons, Patent Attorneys,
50 Queen Street, Melbourne, Victoria, Australia.

DATED this 13th day of September 1984.

HOECHST AKTIENGESELLSCHAFT

by

James Murray

Reg'd Patent Attorney

To:

THE COMMISSIONER OF PATENTS.
In support of the Convention application made under Part XVI of the Patents Act 1952 by HOECHST AKTIENGESELLSCHAFT of 45, Brünningstrasse, D-6230 Frankfurt/Main 80, Federal Republic of Germany for a patent for an invention entitled: "L-AMINOACID OXIDASE FROM YEASTS OF THE GENUS CRYPTOCOCCUS, THEIR PREPARATION AND USE"

We, Karl-Heinrich Meyer-Dülheuer, 31 Höhenstraße, D-6242 Kronberg/Taunus, and Franz Lapice, 2 Sandweg, D-6233 Kelkheim (Taunus); Federal Republic of Germany, do solemnly and sincerely declare as follows:

1. We are authorized by HOECHST AKTIENGESELLSCHAFT the applicant for the patent to make this declaration on its behalf.

2. The basic application as defined by Section 141 of the Act was made in the Federal Republic of Germany under No.P 33 33 453.6 on September 16, 1983 by HOECHST AKTIENGESELLSCHAFT.

3. a) Werner Aretz, 2 Am Krummorg-, D-6233 Kelkheim (Taunus)  
   b) Klaus Sauber, 35 Falkenstraße, D-6232 Bad Soden am Taunus  
a) and b) Federal Republic of Germany

are the actual inventor(s) of the invention and the facts upon which HOECHST AKTIENGESELLSCHAFT is entitled to make the application are as follows:

The said HOECHST AKTIENGESELLSCHAFT is the assignee of the said Werner Aretz and Klaus Sauber.

4. The basic application referred to in paragraph 2 of this Declaration was the first application made in a Convention country in respect of the invention the subject of the application.

DECLARED at Frankfurt/Main, Federal Republic of Germany this 21st day of August 1984

To the Commissioner of Patents

Hoechst
Aktiengesellschaft

[Signatures]

PAT 510
(ppa.Meyer-Dülheuer) (i.V.Lapice)
L-AMINOACID OXIDASE FROM YEASTS OF THE GENUS CRYPTOCOCCUS, THEIR PREPARATION AND USE

HOECHST AKTIENGESELLSCHAFT

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WERNER ARETZ AND KLAUS SAUBER
WM

Claim

1. L-Aminoacid oxidase from yeasts of the genus Cryptococcus.

9. The use as claimed in claim 8, where the L-amino-
oxidase is used as a concentrate from the cytoplasmic membrane or in the form of immobilized cells.

10. Cryptococcus laurentii DSM 2762.
COMMONWEALTH OF AUSTRALIA
PATENTS ACT 1952-69

COMPLETE SPECIFICATION
(ORIGINAL)

Application Number: 33069/84
Lodged: Complete Specification Lodged: 
Accepted: 
Published: 

Class Int. Class

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Address for Service: EDWD. WATERS & SONS,
50 QUEEN STREET, MELBOURNE, AUSTRALIA, 3000.

Complete Specification for the invention entitled:
L-AMINOACID OXIDASE FROM YEASTS OF THE GENUS
CRYPTOCOCCUS, THEIR PREPARATION AND USE

The following statement is a full description of this invention, including the best method of performing it known to: US
The invention relates to a new L-aminoacid oxidase having a broad spectrum of substrates, its isolation by fermentation of yeasts of the genus cryptococcus and its use for the preparation of α-ketoacids, their esters and ethers from the corresponding L-α-aminoacids or their derivatives.

L-Aminoacid oxidase, called LAO below, is an inducible enzyme in yeasts of the genus cryptococcus. Thus, to prepare it, the yeasts are fermented with the addition of, as an inducer, an aminoacid or a substance which releases aminoacids. Preferred embodiments of the invention are illustrated in detail below:

The preferred species of genus cryptococcus is C. Laurentii, for example the strain Cryptococcus laur- tii var. magnus CBS 569, and the species C. albidos. The strain C. Laurentii DSM 2762 is particularly preferred. The starting material for this strain was a sample of soil from Bobodiovlassio (Upper Volta) which was incubated with several transfers, for 2-3 days each time, in a mineral medium containing D-glutamic acid as the only source of nitrogen, at 28°C. These liquid cultures were plated out onto media which contained the ethylamide of D-α-aminoacidic acid as the only N source. After further transfers, the strain DSM 2762, inter alia, was isolated as a pure culture.

This strain is a unicellular, oval yeast which
forms neither mycelium nor pseudomycelia. Multiplication
takes place by manifold budding; the presence of ascospores
or ballistospores has not been detected. The convex,
whitish colonies are rough and have a smooth margin. No
pigment in the form of carotenoids is produced. Yeast
starch was detected with iodine/potassium iodide, both
in the colonies and in the liquid cultures. Physiological
investigations showed that glucose, sucrose, ma"teose,
raffinose, galactose, lactose, starch, rhamnose, melibiose,
dextrin and inositol are assimilated as carbon sources; anaerobic
fermentation of the sugars does not take place. Utilization
of ammonium sulfate, α-aminoadipic acid, glutamic
acid, alanine, leucine, serine, tryptophan, tyrosine and
phenylalanine as nitrogen sources has been demonstrated.
In contrast, growth with sodium nitrate has not been ob-
served.

It has been found that LAO is formed in parallel
with the growth and reaches its highest activity toward
the end of the logarithmic phase. Preferred inducers are
D-aminoacids, especially D-Leu, D-ß-aminoadipic acid
(DαAAA) and D-Ala. A survey of the LAO activities found
is shown in Table 1:
Table 1

Induction of L-aminoacid oxidase by various aminoacids.

<table>
<thead>
<tr>
<th>N source</th>
<th>OD 546 nm</th>
<th>LAO activity u/g cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>9.5</td>
<td>0</td>
</tr>
<tr>
<td>D-Ala</td>
<td>11.9</td>
<td>1.22</td>
</tr>
<tr>
<td>DL-Ala</td>
<td>11.8</td>
<td>0.93</td>
</tr>
<tr>
<td>D- ω AAA</td>
<td>9.3</td>
<td>1.50</td>
</tr>
<tr>
<td>L- ω AAA</td>
<td>9.0</td>
<td>1.25</td>
</tr>
<tr>
<td>DL- ω AAA</td>
<td>8.9</td>
<td>1.76</td>
</tr>
<tr>
<td>D-Lby</td>
<td>5.0</td>
<td>3.70</td>
</tr>
<tr>
<td>L-Leu</td>
<td>6.2</td>
<td>1.20</td>
</tr>
<tr>
<td>DL-Leu</td>
<td>4.2</td>
<td>1.60</td>
</tr>
<tr>
<td>L-Met</td>
<td>3.1</td>
<td>0.70</td>
</tr>
<tr>
<td>DL-Phe</td>
<td>3.6</td>
<td>0.74</td>
</tr>
<tr>
<td>L-Try</td>
<td>3.9</td>
<td>0.50</td>
</tr>
<tr>
<td>L-Ser</td>
<td>13.3</td>
<td>0.30</td>
</tr>
<tr>
<td>D-Glu</td>
<td>10.2</td>
<td>0</td>
</tr>
<tr>
<td>DL-Glu</td>
<td>15.7</td>
<td>0</td>
</tr>
</tbody>
</table>

* Test substrate L-ω-aminoadipic acid (LωAAA)
Preferred C sources are soluble starch and, in particular, lactose and sucrose.

In contrast to the known microbial L-aminoacid oxidases, the LAO according to the invention has a broad spectrum of substrates: apart from most natural amino-acids, other amino-acids such as L-α-aminoacidipic acid and L-cephalosporin C are converted into the corresponding α-ketoacids. However, in addition derivatives of the aminoacids are also converted, namely their esters, especially lower alkyl esters and benzyl esters, as well as the ethers, both ethers of the alcohol group of serine and the phenolic hydroxyl group of tyrosine, and thio-ethers of cysteine. Again, the lower alkyl and benzyl ethers or thioethers are preferred for this. The natural thioether L-methionine is likewise converted.

All the conversions are strictly stereospecific: the L-forms are converted into the corresponding ketoacids or ketoacid derivatives. Thus, according to the invention, it is also possible to resolve racemates, the L-form being converted to the keto derivative while the D-form remains unchanged.

The conversion of the L-aminoacids is advantageously carried out in a pH range from 6.5 to 8.5, advantageously 7-8, in particular 7.5. Thus, suitable buffers are potassium phosphate and tris.HCl buffers.

Advantageous temperatures for the conversion are about 30 to 60, preferably 40 to 55, in particular 50 °C.

The LAO has a Km value of 0.25 mM and a Vmax of
2 mM for L-α-AAA.

The LAO according to the invention is distinguished by high stability on storage. At 4°C, it is utilisable for several days, and at -18°C, it is utilisable without loss of activity for several months.

The LAO according to the invention is localized on the outer cytoplasmic membrane. The enzyme activity is thus equally high in intact cells which have not been made permeable and in cells treated with cetyltrimethylammonium bromide. Freezing and thawing the cells brings about an activity increase of about 30 to 40%.

The LAO according to the invention can be used as a concentrate from the cytoplasmic membrane. However, the use in the form of immobilized cells is particularly advantageous. Since, as mentioned above, the enzyme is localized on the outer cytoplasmic membrane, it is unnecessary to maintain non-toxic conditions when immobilizing the cells.

In addition to the known advantage of enzyme immobilization — increased stability and ease of manipulation — when the whole cells are embedded, isolation and purification of the enzyme is dispensed with.

The immobilization of the enzyme or of the cells can be carried out in a known manner using natural or synthetic polymers (Nachr. Chem. Tech. Lab. 29 (1981) 850, German Offenlegungsschriften 2,252,815, 2,343,633, 2,414,128, 2,420,102 and 2,805,607).

Particularly preferred embodiments of the invention
are illustrated in detail in the examples which follow:

**Example 1**

The yeast Cryptococcus albidus is maintained on the following solid nutrient media:

- **"nutrient broth"** 8 g
- **agar** 15 g
- **dist. water** 1 liter

The medium is distributed over test tubes and sterilized at 121°C for 30 min., then cooled, inoculated with the culture and incubated at 25°C for 3-4 days. The grown culture is rinsed off with 10 ml of sterile saline solution and added to a culture medium of the following composition:

- **glucose** 10 g
- **D-α-AAA** 0.3 g
- **KH₂PO₄** 0.875 g
- **K₂HPO₄** 0.125 g
- **NaCl** 0.1 g
- **MgCl₂.7H₂O** 0.5 g
- **CaCl₂.7H₂O** 0.1 g
- **trace element solution** 1 ml
- **vitamin solution** 10 ml
- **dist. H₂O (pH 7.2)** 1 liter

**Trace element solution:**

- **CoCl₂.6H₂O** 0.25 g
- **NiCl₂.6H₂O** 0.01 g
- **CuCl₂.2H₂O** 0.01 g
- **ZnCl₂** 0.1 g

**Vitamin solution:**

- **biotin** 0.001 g
- **vitamin B₁₂** 0.005 g
- **thiamine·HCl** 0.03 g
- **nicotinic acid** 0.035 g
H₃BO₃ 0.5 g p-aminobenzoic acid 0.02
Na₂MoO₄·2H₂O 0.3 g pyridoxal.HCl 0.01
NaSeO₃·3H₂O 0.1 g Ca pantothenate 0.01
FeSO₄·7H₂O 0.2 g 50% ethanol 1 liter
5 dist. H₂O 1 liter

(adjusted to pH 2-3 with HCl).

500 ml of this medium are placed in 2 liter conical flasks and sterilized at 121°C for 30 min.

The flasks inoculated with a 10 ml inoculum are then incubated at 26°C and 190 rpm in a rotary shaker. After 72 hours, the grown culture is harvested, washed and taken up in a potassium phosphate buffer (pH 7.5, 50 mM). The LAO activity of the intact cells was determined as 1.34 U/g cells using L-κ-AAA as the substrate in an assay dependent on o-phenylenediamine peroxidase.

Example 2

Cryptococcus laurentii DSM 2762 was cultured by the method of Example 1 in 500 ml of nutrient solution and, after 3 days, transferred into a 12 liter fermenter containing 9 liters of the same medium which, however, contained D-leucine in place of D-κ-AAA, and was incubated at 28°C, 400 rpm and an aeration rate of 400 liters of air per hour.

After 4 days, the LAO activity was measured to be 3.5 U/g cells.

Example 3

A 6% strength solution of κ-carrageenan (Marine Colloids, Rockland, Maine, U.S.A.) is made up at 75°C,
cooled to 40°C and mixed with a 4% strength suspension of cryptococcus cells in physiological saline solution in the ratio 1:1. This suspension is injected through a cannula into a precipitation bath (10 mM CaCl₂, 300 mM KCl) so that beads are formed. After stirring for one hour, the product is washed with 0.3 M KCl three times. The carrageenan beads are stored at 4°C in 0.13 M potassium phosphate buffer (pH 7.5) containing 0.02% sodium azide. The activity of the beads is about 50 kU/g wet weight of catalyst.

Example 4

10 μl of 10 mM L-phenylalanine, dissolved in 0.1 M potassium phosphate buffer (pH 8.0) are reacted, passing in air at 37°C, with 4 g of Cryptococcus laurentii PM 2762 cells immobilized by the method of Example 3. Addition of 10 μl of technical catalase (Boehringer, Mannheim) brings about the destruction of the resulting hydrogen peroxide and prevents impairment of product quality. The disappearence of the substrate and the formation of the product can be followed by thin-layer chromatography. The product can be detected by spraying the thin-layer chromatogram with 2,4-dinitrophenylhydrazine. Likewise, the formation of ammonium ions can be followed by the nitroprusside method.

The starting material is quantitatively reacted after 5 hours.

The results listed in Tables 2 and 3 below were obtained by the method of Example 4. Unless otherwise
indicated, the substrate concentration was 4 mM.

Table 2

Substrate spectrum of the LAO from Cryptococcus laurentii DSM 2762

<table>
<thead>
<tr>
<th>Substrate</th>
<th>LAO activity in %</th>
<th>Substrate</th>
<th>LAO activity in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-α-AAA</td>
<td>100</td>
<td>D-Ala</td>
<td>0</td>
</tr>
<tr>
<td>L-Ala</td>
<td>72</td>
<td>D-α-AAA</td>
<td>0</td>
</tr>
<tr>
<td>L-Arg</td>
<td>78</td>
<td>D-Leu</td>
<td>0</td>
</tr>
<tr>
<td>L-Asn</td>
<td>68</td>
<td>D-Meth</td>
<td>0</td>
</tr>
<tr>
<td>L-Asp</td>
<td>&gt; 0</td>
<td>D-Phe</td>
<td>0</td>
</tr>
<tr>
<td>L-Cys</td>
<td>&gt; 0</td>
<td>D-Try</td>
<td>0</td>
</tr>
<tr>
<td>L-Glu</td>
<td>49</td>
<td>D-Val</td>
<td>0</td>
</tr>
<tr>
<td>L-Gly</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Ile</td>
<td>40</td>
<td>L-CPC</td>
<td>76</td>
</tr>
<tr>
<td>L-Leu</td>
<td>73</td>
<td>D-CPC</td>
<td>0</td>
</tr>
<tr>
<td>L-Lys</td>
<td>34</td>
<td>L-Met amide</td>
<td>0</td>
</tr>
<tr>
<td>L-Met</td>
<td>58</td>
<td>L-Leu amide</td>
<td>0</td>
</tr>
<tr>
<td>L-Phe</td>
<td>72</td>
<td>L-Try amide</td>
<td>0</td>
</tr>
<tr>
<td>L-Pro</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Ser</td>
<td>46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Thr</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Try</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Tyr</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Val</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate</td>
<td>LAO activity in %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-α-AAA</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Ala-OMe</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Ala-OEt</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Ala-OBu</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Arg-OMe</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Leu-OMe</td>
<td>100</td>
<td></td>
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</tr>
<tr>
<td>L-Lys-OMe</td>
<td>109</td>
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<td></td>
</tr>
<tr>
<td>D,L-Met-OMe</td>
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<td></td>
</tr>
<tr>
<td>L-Met-OEt</td>
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<tr>
<td>L-Phe-OMe</td>
<td>116</td>
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<tr>
<td>L-Phe-OEt</td>
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<tr>
<td>L-Phe-OtBu</td>
<td>69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Ser-OMe</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Ser-OBz</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-(S-Bz)-Cys*</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-(S-Bz)-Cys-OBz**</td>
<td>63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Tyr Me ether**</td>
<td>74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D,L-Val-OMe</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-1-naphthylalanine</td>
<td>63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-2-naphthylalanine</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 3 mM  
** 2 mM  
*Et = ethyl  
*tBu = tert.-butyl  
*Me = methyl  
*Bz = benzyl
CLAIMS
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. L-Aminoacid oxidase from yeasts of the genus Cryptococcus.
2. L-Aminoacid oxidase from yeasts of the species C. laurentii.
3. L-Aminoacid oxidase from C. laurentii DSM 2762.
4. A process for the preparation of the L-aminoacid oxidase as claimed in claim 1 to 3, which comprises fermenting the said yeasts with the addition of an aminoacid or a substance which releases an aminoacid.
5. The process as claimed in claim 4, wherein a D-aminoacid is used.
6. The process as claimed in claim 5, wherein D-Leu, D-Ala, D-α′-aminoacidic acid or D-β′-aminoacidic acid -γ- semicarbazide are used.
7. The process as claimed in one or more of claims 4 to 6, wherein the carbon source used is lactose or sucrose.
8. The use of the L-aminoacid oxidase as claimed in claim 1 to 3, or of the products obtainable as claimed in claim 4 to 7, for the preparation of α′-ketoacids and their esters and ethers from the corresponding L-α′-aminoacids or derivatives.
9. The use as claimed in claim 8, where the L-aminoacid oxidase is used as a concentrate from the cytoplasmic membrane or in the form of immobilized cells.
10. Cryptococcus laurentii DSM 2762.

DATED this 13th day of September 1984.

Hoechst Aktiengesellschaft

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PATENT ATTORNEYS
50 QUEEN STREET
MELBOURNE, VIC. 3000.