

Process for the preparation of 2-hydroxy-4-methylthiobutyric acid using a nitrilase.

The present invention relates to a new process for the preparation of 2-hydroxy-4-

5 (methylthio)butanoic acid (HMTBA) and/or its ammonium salt (HMTBS).

2-Hydroxy-4-methylthiobutyric acid and its salts have been used for a long time in animal nutrition as a replacement for methionine. It has or
10 they have the advantage over the latter of being in a liquid form, which facilitates their use by feed producing companies.

It has been known for a long time to prepare 2-hydroxy-4-(methylthio)butanoic acid by a chemical
15 route. There can thus be mentioned Patents EP-142 488, EP-143 000 which describe the hydrolysis of 2-hydroxy-4-methylthiohydroxybutyronitrile (HMTBN) by a two-stage process. The first stage consists in bringing the
2-hydroxy-4-methylthiobutyronitrile into contact with a
20 strong inorganic acid such as hydrochloric or sulphuric acid. In a subsequent stage, after dilution with water, the hydrolysis is completed at a higher temperature. The 2-hydroxy-4-(methylthio)butanoic acid is then
extracted with an organic solvent which is not very
25 miscible with water, such as a ketone, preferably methyl isobutyl ketone and then the solvent is removed by evaporation. This type of process, which is used

industrially, nevertheless comprises some disadvantages. It produces a molar quantity of ammonium sulphate which is at least equal to the molar quantity of nitrile introduced which has to be removed, thus
5 generating an industrial waste which runs counter to an environmental protection policy. This process also requires the use of large quantities of solvent which must absolutely be recycled.

Other processes such as those described in
10 Patents US 3,773,927 and 4,353,924 consist in hydrolysing 2-hydroxy-4-methylthiobutyronitrile with hydrochloric acid and then in concentrating the medium with separation of the ammonium chloride formed. The salt obtained is as difficult to remove as the
15 preceding salt and furthermore, the acid obtained has a strong colour.

A process for the chemical hydrolysis of methylthiohydroxypropionitrile which consists, as above, in carrying out a hydrolysis in a sulphuric
20 medium, is again described in Patent EP 330 521. The mixture is partially neutralized with ammonium hydroxide. A two-phase separation appears. The organic phase contains most of the 2-hydroxy-4-methylthiobutyric acid and the aqueous phase contains
25 most of the ammonium sulphate produced. The organic solution, after evaporation of the water which it contains, is filtered so as to recover the dissolved ammonium sulphate. The 2-hydroxy-4-methylthiobutyric

acid is then diluted with a small quantity of water and stabilized with a small quantity of sulphuric acid. The aqueous solution, after elimination of the water, makes it possible to obtain ammonium sulphate which is directly marketable. This process partly overcomes the disadvantages of the prior art processes as regards the use of organic solvents, but does not in any way solve the problems linked to the discharge of the inorganic salts.

10 Among the patents relating to the salts of 2-hydroxy-4-methylthiobutyric acid, there may be mentioned Patents US 2,745,745; 2,938,053 and 3,175,000 relating to calcium and/or ammonium salts. The mixture obtained by hydrolysis of the 2-hydroxy-4-methylthiobutyronitrile is treated with calcium hydroxide or carbonate. The calcium sulphate is then precipitated, releasing the ammonium hydroxide which forms the ammonium salt of 2-hydroxy-4-methylthiobutyric acid. Here again, the problem which consists in removing the calcium sulphate remains.

25 A process which consists in carrying out a hydrolysis and an extraction with an organic solvent like in the first prior art document cited, followed by a neutralization of the organic solution with ammonium hydroxide, is again described in Patent Application WO 96/01808. This process, as in the majority of the processes previously described, leads to the formation of at least one mole of ammonium sulphate or chloride

per mole of nitrile introduced and requires the recycling of large quantities of organic solvent. It cannot make it possible to obtain a solution of ammonium salt of 2-hydroxy-4-methylthiobutyric acid at
5 an industrially advantageous cost.

The use of a nitrilase as a catalyst for the hydrolysis of a nitrile group into a carboxyl group is in addition described in WO 96/09403. The process described in this document is however not capable of
10 being used on an industrial scale given the insufficient activity of the microorganisms which synthesize these nitrilases.

It has now been discovered, by virtue of the process of the invention, that by using several
15 specific stages, it was possible to obtain 2-hydroxy-4-methylthiobutanoic acid and/or its ammonium salt by an enzymatic route on an industrial scale, with a high yield, without the use of solvents and without the concomitant production of inorganic salts.

20 The subject of the invention is a process for the preparation of 2-hydroxy-4-methylthiobutyric acid and/or of the ammonium salt of 2-hydroxy-4-methylthiobutyric acid by enzymatic hydrolysis of 2-hydroxy-4-methylthiobutyronitrile, characterized in that:

- 25 a) in a first stage, a biological material having a nitrilase activity is prepared,
b) in a second stage, it is immobilized,
c) in a third stage, the 2-hydroxy-4-

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methylthiobutyronitrile is exposed to the biological material thus immobilized, in order to obtain the ammonium salt of 2-hydroxy-4-methylthiobutyric acid,

d) in a fourth stage, optionally, the salt
5 obtained in stage c) is converted to the corresponding acid, and

e) in a fifth stage, the product obtained in stage c) or d) is concentrated.

The invention will be explained in greater
10 detail in the following description for which reference will be made to the accompanying figures in which:

- Figure 1 represents a schematic representation of an electrodialysis cell used in the optional stage d); Figure 1A represents an
15 electrodialysis cell with a bipolar membrane having three compartments; Figure 1B represents an electrodialysis cell with a bipolar membrane having two compartments.

- Figure 2 is a schematic representation of a
20 plant for carrying out the process according to the invention;

- Figure 3 represents the restriction map of the plasmids pRPA-BCAT1 to 5;

- Figure 4 represents the strategy for
25 sequencing the fragment of 1130 bp containing the DNA sequence (called nitB in the figure) encoding the polypeptide having the nitrilase activity according to the invention. The numbers refer to the identity of the

primers used for the sequencing as well as the notations M13FWD and M13REV;

- Figure 5 represents the restriction map of the plasmids pRPA-BCAT12 and pRPA-BCAT13;

5 - Figure 6 represents electrophoresis on a 10% SDS-PAGE gel showing the expression of DNA sequences according to the invention in the *E. coli* strains BL21(DE3)/pRPA-BCAT12, BL21(DE3)/pRPA-BCAT13, BL21(DE3)/pRPA-BCAT12 + pXL2231, BL21(DE3)/pRPA-BCAT13
10 + pXL2231. Each lane corresponds to a quantity of 10 µg of soluble proteins and an equivalent volume of crude and insoluble fraction;

 - Figure 7 represents electrophoresis on a 10% SDS-PAGE gel showing the expression of the
15 reference DNA sequence according to the invention in the *E. coli* strains DH5α/pRPA-BCAT3, DH5α/pRPA-BCAT6, DH5α/pRPA-BCAT6 + pXL2035, RPA-BIOCAT76. Each lane corresponds to a quantity of 10 µg of soluble proteins and an equivalent volume of crude and insoluble
20 fraction;

- Figure 8 represents the restriction map of the plasmid pRPA-BCAT14;

 - Figure 9 represents electrophoresis on a 10% SDS-PA gel showing the expression of the DNA
25 sequences according to the invention in the *P. putida* strains G2081 (pRPA-BCAT14), G2081 (pRPA-BCAT23), G2081 (pRPA-BCAT24), *A. faecalis* ATCC8750 (pRPA-BCAT14), *A. faecalis* ATCC8750 (pRPA-BCAT23), *A. faecalis*

ATCC8750 (pRPA-BCAT24). Each lane corresponds to a quantity of 10 μ g of soluble proteins and an equivalent volume of crude and possibly insoluble fraction;

- Figure 10 represents the restriction map of the plasmid pCGL 1087;

- Figure 11 represents the restriction map of the plasmid pOS48.7.

The first stage consists in preparing the biological material having a nitrilase activity.

The biological material may consist of an enzymatic solution as such or whole or broken cells having a nitrilase activity.

Advantageously, a nitrilase-expressing microorganism is used.

The nitrilase may be derived especially from a microorganism, in particular a microorganism of the genus *Alcaligenes*, *Rhodococcus* or *Gordona*, especially *Alcaligenes faecalis*, *Rhodococcus* sp. HT 29-7, *Gordona terrae*, preferably the strains described in WO 96/09403.

A microorganism is advantageously used which expresses a nitrilase activity obtained by transferring the genetic information encoding the nitrilase of a parental microorganism into a host microorganism. In particular, in *E. coli*, it is possible to use the coexpression of the chaperon proteins Gro ESL to enhance the performance of the recombinant strain.

To this end, any appropriate expression

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vector, especially a plasmid vector, is used.

The nucleotide sequence used in this case will then be placed under the control of signals allowing its expression in a cell host. The cell host
5 used may be chosen from prokaryotic systems, such as Gram-positive or Gram-negative bacteria or eukaryotic systems, such as for example yeast, fungi or any other system. The signals controlling the expression of the polypeptides are chosen according to the cell host
10 used. To this end, the DNA encoding a nitrilase may be inserted into autonomously replicating vectors inside the chosen host, or integrative vectors of the chosen host. Such vectors will be prepared according to methods commonly used by persons skilled in the art,
15 and the resulting constructs may be introduced into an appropriate host by standard methods such as electroporation.

By way of host microorganisms, there may be mentioned in particular *Alcaligenes faecalis*,
20 *Pseudomonas putida*, *Escherichia coli*, or microorganisms of the genus *Bacillus*, *Corynebacterium*, *Streptomyces*, *Saccharomyces*, *Kluyveromyces*, *Penicillium* and *Aspergillus*.

A preferred vector for the expression in
25 *E. coli* is the plasmid pRPA-BCAT6.

The second stage of the process consists in immobilizing the biological material. This immobilization is advantageously performed in the

presence of a solid support, and makes it possible to obtain solid particles whose size, shape and mechanical resistance can be controlled. It also allows the simultaneous use of polyazetidine polymer and other cross-linking agents.

This immobilization process may involve whole cells or cells which have been made permeable. It may also apply to a cell-free enzymatic solution.

The enzymes used for the immobilization are the nitrilases.

The immobilization process consists in immobilizing the active biological material on a solid support, especially with a particle size of between 1 μm and 3 mm, preferably 10 μm and 2 mm, by means of chemical agents which react with the amine (NH_2 , NH), carboxyl (COOH), hydroxyl (OH), thiol (SH) or amide (CONH_2) functional groups of the biological agent and of the support. These chemical agents also make it possible to insolubilize the biological material and the support in water. The mass obtained is very malleable and may be shaped in order to obtain particles of desired shape and size. The cohesion and hardness of these particles are then obtained by drying.

The biological material to be immobilized may optionally also contain an inactive biological material present in an amount of 0 to 200% by weight. This inactive biological material may be proteins (albumin,

gelatin) or polysaccharides (Chitosan, k-carrageenan, alginate).

The inert support on which the biological material is deposited and the polymer may be composed
5 of hydrophilic or hydrophobic, porous or nonporous, organic or inorganic particles. Among these particles, there may be mentioned, with no limitation being implied:

- ion-exchange resins,
- 10 - alumina,
- synthetic silicas or diatomaceous earth and silica gels,
- zeolites,
- charcoals,
- 15 - water-insoluble proteins such as gluten,
- polysaccharides such as starch.

The inert support may be added in an amount of 0.01 to 500% by weight of biological material and preferably 10 to 200%.

20 The chemical agents used to insolubilize the biological material may be polymers or bifunctionalized molecules which react with the amine (NH_2 , NH), carboxyl (COOH), hydroxyl (OH), thiol (SH) or amide (CONH_2) functional groups. There may be mentioned:

- 25 - polyazetidine polymers,
- polyethyleneimine polymers,
- polyamide polymers,
- isocyanate polymers,

- alginate gels,
- k-carrageenan gels,
- amines such as hexamethylenediamine,
- aldehydes such as glutaraldehyde,
- 5 - carboxylic acids such as adipic acid,
 and
- isocyanates.

The immobilization process may involve one or more of these chemical agents. The chemical agent is
10 added in a concentration of between 1 and 50% by weight relative to the biological material and to the support. A quantity of between 5 and 30% will be preferred in order to obtain sufficiently solid particles which conserve a high activity and which do not have too many
15 problems of internal diffusion.

The duration of a cross-linking treatment is between 0.5 and 24 hours.

The process temperature is generally between 4 and 65°C. A temperature of between 20 and 40°C will
20 be preferred. The temperature used during the immobilization process may also be highly dependent on the stability of the biological material used.

The pH during the immobilization phase is maintained between 5 and 11. A pH of between 6 and 10
25 will be preferred with a preference for alkaline pH values. The pH is also chosen as a function of the resistance of the biological material and will be easily determined by persons skilled in the art.

The forming of the biocatalyst should allow its use in any system, especially in a fixed bed.

A formulation method may be extrusion. To do this, the biological material and the support are
5 cross-linked by adding one or more chemical agents. After treatment, the insoluble mass is recovered by centrifugation or after flocculation and filtration. A dry matter level of at least 10% is preferred. The mass is then extruded. Using this method, vermicelli with a
10 diameter of between 0.3 and 0.5 mm and a length of between 1 and 10 mm are preferably obtained. These vermicelli may be spheronized. The particles obtained are then dried.

Another formulation method may be spray
15 coating. To do this, the biological material is mixed with one or more chemical agents. After reaction, the mixture is sprayed onto the support in the form of a thin layer. Using this method, granules with a mean diameter of between 0.1 and 2 mm are obtained.

20 The particles obtained may then be optionally immersed in a solution of a reducing agent such as sodium borohydride in order to reduce the imine functional groups formed during the cross-linking.

The particles obtained are sufficiently solid
25 and resistant to attrition to be used in a fixed bed, a fluidized bed or a stirred reactor.

The third stage of the process according to the invention consists in using the immobilized

biological material in one or more columns or reactors. The aim of this stage is to be able to continuously produce the ammonium salt of 2-hydroxy-4-methylthiobutyric acid from 2-hydroxy-4-methylthiobutyronitrile.

5 The column(s) or reactor(s) is(are) supplied with a pure or dilute solution of 2-hydroxy-4-methylthiobutyronitrile or a mixture containing 2-hydroxy-4-methylthiobutyronitrile and the ammonium salt of 2-hydroxy-4-methylthiobutyric acid.

10 The column(s) or reactor(s) is/are preferably used at a temperature of between 10 and 60°C and at a pH of between 5 and 9.

 The system used may consist of two or more columns connected to each other in series, with,
15 according to a first embodiment of the invention, the supply of the aqueous 2-hydroxy-4-methylthiobutyronitrile solution at the top of the first column with a simultaneous supply of the other columns with the 2-hydroxy-4-methylthiobutyronitrile solution in a
20 quantity limited to the solubility of this compound in the reaction mixture; this system is called a staged system. According to a second embodiment of the invention, one or more columns connected to each other in parallel in a circulation loop is used. According to
25 this plant, the 2-hydroxy-4-methylthiobutyronitrile in aqueous solution is continuously supplied to the loop, and the reaction medium is continuously pumped so as to conserve a constant volume in the loop; this system is

called loop system.

The type of reactor used in this invention may be of the fixed bed, fluidized bed or continuously stirred bed type. The fixed bed-type reactors are
5 preferably used because they reduce the problems of attrition which may be encountered with the immobilized cell particles. If the microorganism is used as it is, a stirred reactor coupled to an ultrafiltration module to continuously separate the microorganism from the
10 product of interest will be preferably used.

The fourth stage, which is an optional stage, consists in converting the ammonium salt of 2-hydroxy-4-methylthiobutyric acid to the corresponding acid. This stage may be carried out according to two methods:

- 15 - either by electrodialysis by means of a two- or three-compartment electrodialyser,
 - or by heating the aqueous solution which may be followed by a liquid/liquid extraction.

Depending on the electrodialysis method, a
20 two- or three-compartment electrodialyser will be used.

"Compartment" is understood to mean the space which is either between two membranes or between a bipolar and a homopolar membrane, or between two adjacent homopolar membranes. "Cell" is understood to
25 mean a set of two or three compartments. A stack comprises between 5 and 300 cells. The electrodialyser is composed of a stack and of course comprises an anode and a cathode.

The homopolar membranes which may be used within the framework of the invention can be divided into two large families, depending on their mode of manufacture.

5 Thus, heterogeneous membranes, prepared from ion-exchange resins, mixed with a binder such as polyvinyl chloride, polyethylene and the like, may be used. The set thus formed may coat a weft such as for example a polyester or polyacrylonitrile fabric.

10 It is also possible to use homogeneous membranes obtained by introducing a functional group onto an inert support, by chemical or radiochemical grafting. The most widely used chemical method generally consists in functionalizing a latex of a
15 polymer comprising aromatic rings, such as styrene/divinylbenzene or styrene/butadiene. The latex thus functionalized may then serve to coat a weft like for the heterogeneous membranes. The radiochemical method generally comprises the grafting, under the
20 influence of a radiation, of an aromatic compound, such as styrene, onto an inert support such as a polyethylene or polytetrafluoroethylene sheet. The aromatic ring is then functionalized as in the chemical method.

25 The cation-exchange membranes comprise strong acid groups, most often sulphonate groups, or weak acid groups, often carboxylate groups. More rarely, the acidic groups may be PO_2^{2-} , HPO_2^- , AsO_3^{2-} , SeO_3^- .

The anion-exchange membranes comprise strong basic groups, most often quaternary ammonium groups, or weak basic groups, most often amine groups. More rarely, the basic groups may be quaternary phosphonium groups or sulphonium groups.

In the present process, the cationic membranes preferably comprise strong acidic groups and among these, preferably sulphonate groups and the anionic membranes preferably comprise strong basic groups and among these, quaternary ammonium groups.

The bipolar membranes are an assembly of two membranes, one cationic, the other anionic. When the membrane is subjected to an adequate electric field, the water of solvation at the interface of the membrane dissociates into H^+ and OH^- ions which migrate towards the cathode by crossing the cationic face and towards the anode by crossing the anionic face, respectively. As bipolar membranes, there may be mentioned, by way of example, the membranes marketed by the companies Aqualytics, Tokuyama Soda or FuMaTech.

The anode of the electrodialyser may consist of materials conventionally used in electrodialysis, for example graphite, nickel or titanium coated with precious metals or precious metal oxides, especially platinized titanium. The cathode may also consist of materials conventionally used in electrodialysis, for example graphite, stainless steel or nickel.

The electrodialyser is supplied with the

aqueous solution to be treated. It is also necessary to circulate to the anode a solution of an anolyte and to the cathode a solution of a catholyte. A single electrolyte solution may also be used. In the present
5 process, a single electrolyte circuit is quite suitable. The role of the electrolyte solution is to ensure a sufficient conductivity. Preferably, this conductivity will be equal to or greater than
20 millisiemens per centimetre (mS/cm), without this
10 lower limit being considered to be critical for carrying out the process.

The electrolyte used is an ionizable compound such as a salt, an acid or a base. The electrolyte is preferably chosen from nonelectroactive compounds.
15 Thus, for example, it is preferable to use neutral salts such as sulphates, acids such as sulphuric acid, bases such as sodium hydroxide.

The applied current densities are generally between 0.2 and 1.5 kA/m², and preferably between 0.4
20 and 1 kA/m².

The temperature at which the process of the invention is carried out is situated in a range compatible with the stability of the membranes. The procedure will be preferably carried out at a
25 temperature of between 30 and 60°C.

The electrodialyser can work in different ways. It can first of all work continuously, the solution to be treated continuously crossing the stack;

several stages are then placed in series if the level of treatment to be obtained requires it. It can also work batchwise, the solution to be treated recirculating over a tank until the desired treatment level is obtained. Finally, it can work by direct passage with partial recirculation.

According to a first variant of the invention, the dissociation of the ammonium salt into 2-hydroxy-4-(methylthio)butanoic acid and ammonium hydroxide can occur in an electrodialysis cell with bipolar membranes having three compartments, as schematically represented in Figure 1A.

An electrodialysis apparatus suitable for carrying out the process consists of different compartments, respectively delimited by cationic membranes (MC), bipolar membranes (MB) and anionic membranes (MA). These compartments divide into a salt compartment (S) which is depleted of compounds to be separated, into a base compartment (B) and an acid compartment (A) where the acid and the base regenerated from the salt are concentrated respectively.

The ammonium salt is introduced into the salt compartment. Under the action of the electric field, the ammonium ion migrates towards the cathode, leaving the compartment (S) where it is present, across a cation-exchange membrane (cationic membrane) and combines with the OH^- ions from the anionic face of the bipolar membrane, in which the dissociation of the

water occurs under the effect of the electric field.

Simultaneously, the carboxylate (2-hydroxy-4-(methylthio)butanoate) ions migrate towards the anode, leaving the compartment (S) where they are present, across an anion-exchange membrane (anionic membrane). After passing into the next compartment (A), they are protonated through the supply of H^+ ions from the cationic face of the bipolar membrane. The three adjacent compartments (B), (A), (S) form an electro dialysis cell.

In a second variant of the invention, the regeneration of the 2-hydroxy-4-(methylthio)butanoic acid can take place in an electro dialysis cell with bipolar membranes having two compartments as represented in Figure 1B. These two compartments are delimited by cationic membranes and bipolar membranes respectively. These compartments divide into a salt/acid (S/A) and base (B) compartment.

The ammonium salt is introduced into the salt/acid compartment. Under the action of the electric field, the ammonium ion migrates towards the cathode by leaving the compartment (S/A) where it is present, across a cation-exchange membrane (cationic membrane) and combines with the OH^- ions from the anionic face of the bipolar membrane, in which the dissociation of the water occurs under the effect of the electric field.

Simultaneously, the compartment S/A becomes acidified through the supply of H^+ ions from the

cationic fase of the bipolar membrane. The two adjacent compartments (B) and (S/A) form an electrodialysis cell.

This configuration has the advantage of a
5 lower energy consumption and makes it possible to vary the desired salt/acid ratio.

To obtain a good operation of the electrodialyser, the electrical conductivity of the base compartment (as well as that of the acid
10 compartment in the case of the three-compartment configuration) should be adequate and can be adjusted by addition of a support electrolyte. Thus, the conductivity of the ammonium hydroxide solution may be increased by adding an ammonium salt, such as ammonium
15 sulphate.

In a preferred variant of the invention, ammonium 2-hydroxy-4-methylthiobutanoate will be used.

In a specific embodiment which can be envisaged within the framework of the present
20 invention, a mixture of ammonium salt of 2-hydroxy-4-methylthiobutyric acid and 2-hydroxy-4-methylthiobutyronitrile is introduced into the salt compartment of a three-compartment electrodialysis cell. The salt will be dissociated as above into
25 2-hydroxy-4-methylthiobutyrate and will migrate towards the anode where it will be converted to 2-hydroxy-4-methylthiobutyric acid, the ammonium ion will migrate towards the cathode where it will be converted to

ammonium hydroxide, and there will remain in the salt compartment the water and the unconverted 2-hydroxy-4-methylthiobutyronitrile which will be recycled towards the hydrolysis column.

5 Using the heating method, the acid is recovered by shifting the ammonium salt, free acid equilibrium by heating the HMTBS-rich aqueous solution and by withdrawing the ammonium hydroxide thus released. This can be achieved by heating under vacuum
10 or at atmospheric pressure with or without a stripping treatment. The use of CO_2 under pressure may be envisaged in order to facilitate the shifting of the equilibrium. A mixture of HMTBS and HMTBA is obtained in an amount of 5 to 99.9% HMTBA, and preferably 10 to
15 50% HMTBA relative to the sum of HMTBA and HMTBS. The viscosity of these solutions are between 1200 and $30 \text{ mm}^2 \cdot \text{s}^{-1}$ (1200 and 30 cSt) and preferably between 200 and $50 \text{ mm}^2 \cdot \text{s}^{-1}$ (200 and 50 cSt) at 25°C .

 The aqueous solutions may be decomposed by
20 extraction of the acid with the use of partially water-miscible or water immiscible solvent products. A large number of possible solvents exist for the separation. The preferred solvents are C_5 to C_8 ketones, in particular methyl isobutyl ketone, esters such as
25 isopropylether, alcohols such as isopropanol, esters, tertiary amines such as trioctylamine. Within the framework of the invention, the preferred solvents are: acetone, MIBK, isopropanol, isopropyl acetate, isobutyl

or propyl ether or THF, trioctylamine. The ratio of the aqueous phase to the organic phase is not critical. However, for reasons of degree of efficacy, it should not fall below a minimum ratio of 1/1 and for reasons of profitability, it should not rise above a ratio of 1/3. A 1/1.5 to 2.0 ratio is preferred.

The extraction may be carried out batchwise or continuously, in any type of liquid-liquid extraction technology. The cascade of co- or contracurrent mixing-decanting devices, centrifugal extractors, packed or plate columns and the like may be mentioned for example.

The aqueous solution depleted of free acid may again be treated, and as many times as desired until a complete depletion of the ammonium hydroxide is obtained if desired.

The organic phase is subjected to a treatment in order to isolate the HMTBS. This is preferably carried out by vaporization of the solvent or by an extraction with hot water. To avoid a possible deterioration of the HMTBA, the heat treatment can be maintained as low as possible by application of a vacuum.

In these two ammonium salt dissociation processes, an aqueous ammonium hydroxide solution is generated. The latter may be treated in order to concentrate the ammonium hydroxide. Distillation and concentration of the ammonium hydroxide in one or more

stages with or without pressure will be preferred. A preliminary stripping stage may be envisaged. The concentrated ammonium hydroxide solution may be returned into the synthesis of the hydrocyanic acid which is involved in the synthesis of 2-hydroxy-4-methylthiobutyronitrile.

In a fifth stage, the aqueous solution of HMTBS and/or of free acid is concentrated. This is preferably carried out by evaporation of the water.

The subject of the invention is also a plant for carrying out the process according to the invention. Such a plant is schematically illustrated in Figure 2 and comprises the conduits 1, 2 intended for introducing the MTPA cyanhydrin and the water, respectively, into the reactor 3. The reactor 3 is a fixed bed with recirculation which is packed with the immobilized enzymes or strains. Part of the solution is withdrawn from the reactor 3 and sent into a finishing reactor 4. The finishing reactor 4 is of the fixed bed type packed with the immobilized enzymes or strains. A concentrated HMTBS solution is then recovered at the outlet of the reactor 4 via the conduit 5.

This concentrated HMTBS solution may be subjected to two completely independent treatments according to the type of final product desired.

If it is desired to recover a product containing a high proportion of HMTBS, the solution from the conduit 5 is conveyed into an evaporator 6

which makes it possible to concentrate the product and a concentrated solution composed mainly of HMTBS and containing a small quantity of free acid is recovered in the conduit 8. The excess water as well as a
5 fraction of the ammonium hydroxide are evacuated by the conduit 7.

If it is desired to recover a product containing a high proportion of free acid, the concentrated HMTBS solution from the conduit 5 is
10 conveyed into a bipolar electrodialysis system 9. In this apparatus, the ammonium hydroxide is separated to a greater or lesser degree from the acid by electrodialysis. The mixture depleted of ammonium is recovered in the conduit 11 and then concentrated in
15 the evaporator 12 in order to obtain a concentrated solution composed mainly of free acid and containing little or no HMTBS. The ammonium hydroxide-rich solution is withdrawn via the conduit 10. The ammonium hydroxide is recovered by stripping 15.

20 This ammonium hydroxide effluent may be concentrated by distillation 16, the water is recovered in 18. The concentrated ammonium hydroxide solution 17 may be returned to the synthesis of HCN.

The following examples illustrate the
25 invention.

EXAMPLES**Example 1: Purification and N-ter. sequencing of nitrilase**

The nitrilase was purified in four stages.

5 The summary of the purification is given in Table 1.

The *Alcaligenes faecalis* cells are cultured for 24 hours, at 30°C, in a minimal medium in the presence of benzonitrile (0.5 g/l). After centrifugation of the culture, the pellet is taken up
10 in TG buffer (25 mM Tris-HCl, 10% (w/v) glycerol, pH 7.5). The cellular suspension is treated with ultrasound and then centrifuged in order to obtain the crude extract. The crude extract is then treated with ammonium sulphate up to 30% saturation. The precipitate
15 obtained is resuspended in TG buffer and then dialysed against 2 litres of the same buffer overnight. The solution obtained is then loaded onto a Q Sepharose Fast Flow HR 26/10 anion-exchange column previously equilibrated with TG buffer. The activity is then
20 eluted with a gradient from 0 to 1 M NaCl. The active fractions are then loaded onto a Mono Q HR 5/5 anion-exchange column previously equilibrated with TG buffer. The nitrilase is eluted by means of a gradient from 0 to 1 M NaCl. To finish, the fractions containing the
25 activity are combined, the ammonium sulphate concentration is then adjusted to 1 M. This solution is then loaded onto a Phenyl Superose HR 5/5 hydrophobic interaction column previously equilibrated with TG

buffer supplemented with 1 M $(\text{NH}_4)_2\text{SO}_4$. The activity is then eluted with a gradient from 1M to 0M ammonium sulphate.

Table 1: Summary of the purification of nitrilase

5	Fraction	Total prot (Mg)	Total activity ($\mu\text{Mol/h}$)	Specific activity ($\mu\text{mole/h.mg}$)	Protein yield (%)	Purification factor
	Whole cells	955	4300	4.5	100	1
	Crude extract		2400			
	Ammo. sulphate		650			
	QSHL 26/10	3	360	120	0.3	27
10	MonoQ HR 5/5	1.5	240	160	0.15	35
	Phenyl sepharose	1	120	110	0.1	24

Operating conditions: [nitrile] = 50 mM; phosphate
15 buffer 100 mM pH 7.0; 30°C.

The molecular weight of the protein is determined by gel filtration. It is about 260 kDa. On an SDS-PAGE gel, a single band of 43 kDa is observed (95% purity). It is therefore probably a protein of α_6
20 structure with α weighing 43 kDa.

Example 2: Cloning of the *Alcaligenes faecalis* ATCC8750 nitrilase

The NH_2 -terminal sequence presented in Example 1 exhibits complete identity with the sequence of the

N-terminal end of the *A. faecalis* JM3 nitrilase (Kobayashi et al., 1993, *Proc. Natl. Acad. Sci. USA* 90: 247-251), whereas the N-terminal ends of bacterial nitrilases exhibit 35 to 57% identity on 14 residues.

5 The inventors emitted the hypothesis that the nitrilase of the invention purified from the ATCC8750 strain was similar to that described by Kobayashi et al. (cited above). The cloning strategy then consisted in amplifying by a PCR reaction on the genomic DNA of the

10 ATCC8750 strain the gene for this nitrilase, with the aid of two nucleotide probes determined from the sequence given by Kobayashi et al. (cited above).

The two probes were synthesized, one being capable of hybridizing with the 5' part of the sequence

15 given by Kobayashi et al. (cited above) and the other with the 3' part:

5' Part (PCRAF1): CCGGGAATTCATATGCAGACAAGAAAAATCGTCC

3' Part (PCRAF2): TCCTTCTGCGTCCCGATCCCGCAT

The primer PCRAF1 comprises 12 nucleotides in

20 5' which make it possible to introduce upstream of the ATG initiation codon the restriction sites of the enzymes *EcoRI* and *NdeI*. The primer PCRAF2 makes it possible to introduce the restriction site of the enzyme *BamHI*. The genomic DNA of the *A. faecalis*

25 ATCC8750 strain was extracted according to the CTAB procedure described by Ausubel et al. (Current

Protocols in Molecular Biology, John Willey & Sons Inc. Ed., 2.4.1-2.4.5) and 100 ng were used for each PCR reaction. In order to vary the specificity of amplification, various $MgCl_2$ concentrations were tested as indicated in Ausubel et al. (cited above) in a total sample volume of 100 μl and with 2.5 units of Taq DNA polymerase (Perkin Elmer). Two additional reactions were carried out with 1.5 mM $MgCl_2$ and 5% DMSO or 5% formamide. A Perkin Elmer 9600 thermocycler was programmed with the following sequence: 5 min at 95°C, 30 cycles (30 sec at 95°C, 30 sec at 55°C, 45 sec at 72°C) and 4 min at 72°C. The different amplification products were analysed on a 0.8% agarose gel. A predominant band, whose size corresponds to the expected size of 1.15 kb, were amplified in all the reactions but more specifically with 1.5 mM $MgCl_2$ and 5% DMSO. The reaction products under these conditions were therefore retained for subsequent stages. The sample was treated with proteinase K, ethanol-precipitated after a phenol-chloroform extraction. The pellet taken up in suspension was incubated for 2 h at 37°C with 40 units of *EcoRI* enzyme and 40 units of *BamHI* enzyme. After running on a 0.7% agarose gel, the 1.15 kb band was cut out and extracted so as to be cloned into the vector pBSK⁻ (Stratagene, La Jolla, USA) opened [*lacuna*] *EcoRI*-*BamHI* using conventional methods. Five independent clones, called pRPA-BCAT1 to 5, were analysed by enzymatic digestion of the plasmid DNA with

the enzymes *NdeI*, *EcoRI*, *BamHI*, *BspMI* and *BglII* (Figure 3). The pattern obtained corresponded to the theoretical restriction pattern for a plasmid obtained by this method with the sequence described by Kobayashi et al. (cited above). The strain XL1Blue (pRPA-BCAT3) was deposited at CBS under the number CBS 998-96.

Example 3: Sequencing of a 1130 bp fragment containing the DNA encoding the polypeptide having the nitrilase activity.

10 The insert cloned into the plasmid pRPA-BCAT3 was sequenced by the company Génome Express S.A. (Grenoble, France) using a DNA preparation produced in the laboratory (Wizzard Midi-prep kit, Promega). The strategy for sequencing this fragment, carried out
15 according to conventional methods known to persons skilled in the art, is indicated in Figure 4. The ten internal nucleotide primers (identified by the numbers 623 to 629 and 681 to 682 in Figure 4) were synthesized based on the sequence of the *A. faecalis* JM3 nitrilase
20 (Kobayashi et al., cited above). This set was supplemented with the universal primers "Reverse" and "M12 Forward". Each region was read at least once on each DNA strand.

 The DNA sequence obtained has two differences
25 relative to the published sequence: one in the structure which is supposed to serve as transcription terminator and the other in the nitrilase gene, called

nitB, leading to the substitution Asn²⁷⁹-->Asp. These two regions were then sequenced in the laboratory on the plasmids pRPA-BCAT1, 2, 4, 5 with two specific primers 710 and 682 (cf. Figure 4). The C→T change at position 1412 (in accordance with the numbering by Kobayashi, cited above) in the terminator was found in all the clones. This is a mutation which differentiates the two *A. faecalis* strains. The A→G change at position 1138 is present only in the plasmid pRPA-BCAT3. This is therefore a mutation introduced during the PCR by the Taq DNA polymerase.

Example 4: Expression of the nitrilase in *E. coli* BL21(DE3).

In order to confirm the identification of the DNA sequence cloned with the purified nitrilase gene, the nitB gene was placed under the control of the T7 phage (PT7) ϕ 10 gene promoter according to the procedure described below: the 1.13 kb NdeI-BamHI inserts of the plasmids pRPA-BCAT3 and pRPA-BCAT4 were cloned into the vector pXL2432 to give respectively the vectors pRPA-BCAT12 and pRPA-BCAT13 described in Figure 5. The parent vector pXL2432 is a hybrid between the region of the plasmid pET9 (Studier et al., 1990, Methods in Enzymol. 185, 60-89), between the AccI and EcoRI sites, which includes the replication origin (ORI) and the selectable marker during the kanamycin (Kan) resistance, and the region between the EcoRI and

AccI sites of the plasmid pET11a (Novagen Inc., Madison WI, USA) which includes the expression cassette, the repressor gene lacI and the gene regulating copy number ROP.

5 Cultures were carried out under induction conditions according to the procedure below: the strain BL21(DE3) (Novagen Inc., Madison WI, USA) containing the plasmid pRPA-BCAT12, the strain BL21(DE3) containing the plasmid pRPA-BCAT13 as well as the
10 strain BL21(DE3) containing the plasmid pXL2432 were cultured for 16 h in LB medium at 37°C (Miller, 1972, Experiments in Molecular Genetics - Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) containing 50 µg/ml of kanamycin, and then diluted 1/100 in the
15 same medium and at the same temperature. When the cultures reach an OD₆₀₀ of between 0.5 and 1, IPTG, at the final concentration of 1 mM, was added. After culturing for 6 hours, the bacteria were collected. A similar procedure was adopted to culture the strain
20 BL21(DE3) containing the plasmids pRPA-BCAT12 and pXL2231, the strain BL21(DE3) containing the plasmids pRPA-BCAT13 and pXL2231 as well as the strain BL21(DE3) containing the plasmids pXL2432 and pXL2231 by adding to the culture medium tetracycline in an amount of
25 12 µg/ml of medium. The plasmid pXL2231, which is derived from the vector pXL1635 (Application FR 90/05185 of 24/04/1990), belongs to the IncP incompatibility group and is therefore compatible with

the plasmids pRPA-BCAT12 and 13 which possess the replication origin of the plasmid ColE1. Its selectable marker is tetracycline resistance and it carries a 2.2 kb *EcoRI-HindIII* fragment containing the GroES and GroEL genes encoding the *E. coli* molecular chaperons (Fayet et al., 1986, Mol. Gen. Genet. 202: 435-445).

The expression of the nitrilase was analysed on a 10% SDS-PA gel in the crude fraction after sonication of the cells, and after centrifugation in the pellet and in the supernatant. The results are presented in Figure 6 and show a high nitrilase (NitB) expression level in the extracts of cells in which at least one of the plasmids contains the *nitB* insert; however, this protein is essentially in insoluble form although the presence of the plasmid pXL2231 makes it possible to increase the quantity of nitrilase polypeptide in the soluble fraction.

In Figure 6, M represents the molecular weight marker; these are indicated in kDa. Moreover, the lanes have the meanings below:

- A, D, G represent the crude fractions of BL21(DE3) + p/RPA-BCAT12/RPA-BCAT13/XL2432 respectively;
- B, E, H represent the supernatants obtained respectively from these same strains;
- C, F, I represent the pellets obtained respectively from these same strains;
- J, N, Q represent the crude fractions

obtained respectively from

BL21(DE3) + pXL2231 + p/RPA-BCAT12/RPA-BCAT13/XL2432:

- K, O, R represent the supernatants obtained respectively from these strains;

5 - L, P, S represent the pellets obtained respectively from these strains.

The strain BL21(DE3)/pRPA-BCAT12 + pXL2231 was called RPA-BIOCAT126. The strain BL21(DE3)/pRPA-BCAT13 + pXL2231 was called RPA-BIOCAT127. The
10 nitrilase activities of the cultures of RPA-BIOCAT126, RPA-BIOCAT127, and RPA-BIOCAT66 which corresponds to BL21(DE3)/pXL2432, were determined as follows: the cultures were carried out according to the procedure described above, with modification of the culture
15 volume (50 ml) and the final concentration of IPTG (0.1 mM). The cultures were centrifuged and the cellular pellets taken up in 10 ml of 100 mM potassium phosphate buffer pH 7. The hydrolysis of HMTBN was carried out with 500 μ l of this suspension added to
20 500 μ l of 200 mM HMTBN solution pH7. A kinetic of hydrolysis is obtained by mixing 100 μ l of this mixture with 900 μ l of 0.1 N phosphoric acid at regular intervals for 1 to 4 hours. The quantities of HMTBA produced are analysed by HPLC as described in
25 Application WO 96/09403. The results are assembled in Table 2.

Table 2: Activities of the strains RPA-BIOCAT66, 126 and 127

RPA-BIOCAT	MEDIUM	INDUCTION	CULTURE TIME	OD ₆₆₀	ACTIVITY (U)
66	LB Km	0.1 mM IPTG	24 h	2.5	0
126	LB Km Tc	0.1 mM IPTG	24 h	2.4	15
127	LB Km Tc	0.1 mM IPTG	24 h	1.9	10

ABBREVIATIONS: Km: Kanamycin 50 µg/ml; Tc: tetracycline
12 µg/ml; h: hours; U: kg of HMTBA formed per
hour and per kg of dry weight.

In order to enhance the solubilization of the nitrilase polypeptide expressed, the plasmid pRPA-BCAT37 was constructed as below. The plasmid pXL2391 was obtained by ligating the 5.9 kb *EcoRI*-*PvuII* fragment of the plasmid pDSK519 (Keen et al., 1988, Gene 70: 191-197), treated with Klenow polymerase, with the 2056 bp *HindIII* fragment extracted from the plasmid pHP45ΩSp (Prentki and Krisch, 1984, Gene 29: 303-313) treated with Mung Bean nuclease. The plasmid pXL2391 was then digested with *SmaI* and *SacI* and the 2.3 kb insert carrying the GroESL operon, extracted from the plasmid pXL2231 digested with *HindIII*, treated with Klenow and digested with *SacI*, was introduced therein. The plasmid pRPA-BCAT37 is therefore a derivative of the plasmid RSF1010 with a higher copy number than the

plasmid pXL2231, compatible with the plasmids carrying the origin ColE1 and carrying a marker for resistance to streptomycin. This plasmid was introduced into the strain BL21(DE3)/pRPA-BCAT12 to give the strain RPA-BIOCAT171. The activity of a culture carried out according to a procedure similar to that described above, but replacing the tetracycline with streptomycin at 100 $\mu\text{g/ml}$, was determined and is reported in Table 3.

Table 3: Activity of the strain RPA-BIOCAT171

RPA-BIOCAT	MEDIUM	INDUCTION	CULTURE TIME	OD ₆₆₀	ACTIVITY (U)
171	LB Km Sm	0.1 mM IPTG	24 h	3.2	14

ABBREVIATIONS: Km: Kanamycin 50 $\mu\text{g/ml}$; Sm: Streptomycin 100 $\mu\text{g/ml}$; h: hours; U: kg of HMTBA formed per hour and per kg of dry weight.

Example 5: Expression of the nitrilase in *E. coli* DH5alpha.

The plasmid pRPA-BCAT6 was constructed by cloning into pBCAT3 (cf. Figure 3) the 0.6 kb *ScaI*-*NdeI* fragment of pXL2158 containing the *P_{trp}* promoter and the ribosome-binding site RBS_{CI} (Levy-Schill et al., 1995, Gene 161: 15-20). The expression was performed with the strain DH5alpha containing the plasmid pRPA-BCAT6 and/or the plasmid pXL2035 (Levy-Schill et al.,

cited above) with the following procedure: the preculture is incubated for 16 h at 37°C in an M9 glucose medium (Miller, J.H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) containing 0.4% of casamino acids, 100 µg/ml of tryptophan, 100 µg/ml of carbenicillin. For the strains containing pXL2035, kanamycin is added in an amount of 50 mg/l. The expression occurs after 1/100 dilution of the saturated culture in an identical medium but without tryptophan and incubation for 8 to 16 h at 37°C.

SDS-PAGE analysis of the extracts of the different strains is performed as described in the preceding example and is represented in Figure 7.

In this figure, M represents the molecular weight marker; these are indicated in kDa. Moreover, the lanes have the meanings below:

- L, I, F, C represent the crude fractions obtained respectively from the strains DH5alpha + pRPA-BCAT/3,/6,/6 + pXL2035, RPA-BIOCAT76;

- K, H, E, B represent the supernatants obtained respectively from these same strains;

- J, G, D, A represent the pellets obtained respectively from these same strains.

The plasmid pRPA-BCAT6 makes it possible to obtain a small accumulation of a predominantly insoluble 43 kDa polypeptide. The coexpression of GroE from the plasmid pXL2035 reduces the overexpression,

but after three successive subculturings of the strain DH5alpha (pRPA-BCAT6 + pXL2035), the strain RPA-BIOCAT76 selected returns to an initial level of expression of the nitrilase polypeptide, the latter being practically completely soluble. The assays of activities of the cultures carried out according to the procedures described above and in the preceding example are presented in Table 4.

Table 4: Activity of the strains DH5alpha (pRPA-BCAT3), DH5alpha (pRPA-BCAT6), DH5alpha (pRPA-BCAT6 + pXL2035), RPA-BIOCAT76

STRAIN	MEDIUM	CULTURE TIME	OD ₆₆₀	ACTIVITY (U)
DH5alpha (pRPA-BCAT3)	M9 Cb	24 h	4	0
DH5alpha (pRPA-BCAT6)	M9 Cb	24 h	4	0.6
DH5alpha (pRPA-BCAT6 + pXL2035)	M9 Cb Km	24 h	3	1.6
RPA-BIOCAT76	M9 Cb Km	24 h	3.8	5

ABBREVIATIONS: Km: Kanamycin 50 µg/ml; Cb: Carbenicillin 100 µg/ml; h: hours; U: kg of HMTBA formed per hour and per kg of dry weight.

These data show that the coexpression of GroE makes it possible to enhance the expression of the nitrilase and that a conventional strain selection by

successive subculturing also contributes towards the enhancement of the activity of the recombinants.

Example 6: Expression of the nitrilase in *Pseudomonas putida* and *Alcaligenes faecalis*.

5 The expression system described in Example 5 was used to produce the nitrilase in *Pseudomonas putida* and *A. faecalis*. The 1.14 kb NdeI-XbaI fragment of pRPA-BCAT6 containing the *nitB* gene was introduced into the NdeI-XbaI sites of the vector pXL1289. The vector
10 pXL1289 is a derivative of pKT230 (Bagdasarian et al., 1981, Gene 15: 237-247) carrying the multihost replicati~~oin~~ origin (*oriV*) in Gram-negative bacteria and which contains a 120 bp EcoRI-NdeI fragment carrying the *P_{trp}* promoter and RBScII of pXL534 (Latta
15 et al., 1990, DNA and Cell Biology, 9: 129-137), an NdeI-XbaI insert encoding the *cobA* gene (Crouzet et al., 1990, J. Bacteriol. 172: 5968-5979) and a 500 bp XbaI-BamHI insert containing the *T_{rrmB}* terminator of pXL534 (Latta et al., 1990, DNA and Cell Biology, 9:
20 129-137). The plasmid obtained pRPA-BCAT14 is therefore a derivative of pKT230 containing a gene which confers resistance to kanamycin (Kan) and the *nitB* gene under the control of *P_{trp}*:RBScII (Figure 8).

 During the introduction of this plasmid into
25 the *E. coli* DH5alpha strain, a specific clone was selected: it expressed a nitrilase whose molecular weight on an SDS-PA gel is 44 kDa instead of 43 kDa.

The plasmid harboured by this clone exhibits the same restriction pattern as pRPA-BCAT14 and was called pRPA-BCAT24. Finally, the plasmid pRPA-BCAT23 was constructed according to the same procedure as pRPA-BCAT14 with the following modification: the 136 bp *StuI*-*BsmI* fragment of pRPA-BCAT6 was replaced by the *StuI*-*BsmI* fragment of pRPA-BCAT4. The plasmid pRPA-BCAT23 therefore expresses a nitrilase *NitB* with an Asn residue at position 279.

10 The plasmids pRPA-BCAT14, 23 and 24 were introduced by electroporation into the *Pseudomonas putida* G2081 strain. The strain G2081 is derived from the strain KT2440 (Bagdasarian and Timmis, 1981, in Hofschneid and Goebel, Topics in Microbiology and Immunology, 47, Springer Verlag, Berlin) by selection of the spontaneous resistance to nalidixic acid and to rifampycin. The vector pKT230 was used as control plasmid.

20 The plasmids pRPA-BCAT14, pRPA-BCAT23, pRPA-BCAT24 and pKT230 were then extracted from the *P. putida* strains so as to be introduced by electroporation into the *A. faecalis* ATCC8750 strain (= RPA-BIOCAT1).

25 The strains G2081 (pRPA-BCAT14), G2081 (pRPA-BCAT23), G2081 (pRPA-BCAT24), G2081 (pKT230) with RPA-BIOCAT1 (pRPA-BCAT14), RPA-BIOCAT1 (pRPA-BCAT23), RPA-BIOCAT1 (pRPA-BCAT24), RPA-BIOCAT1 (pKT230) were cultured overnight at 30°C in LB medium containing

50 mg/l of kanamycin. These precultures were diluted 1/100 in M9 medium containing 50 mg/l of kanamycin and incubated for 20 h at 30°C.

After sonication of the cells, the expression of the nitrilase was measured on a 10% SDS-PA gel in the crude fraction after sonication of the cells, and after centrifugation in the pellet and in the supernatant. The results are presented in Figure 9. For the strains RPA-BIOCAT1 (pKT230) and G2081 (pKT230), only the crude extracts were loaded (lanes A and I respectively). In this figure, M represents the molecular weight marker; these are indicated in kDa. Moreover, the lanes have the meaning below:

- B, D, F represent the crude fractions obtained respectively from the strains RPA-BIOCAT1 (pRPA-BCAT14), RPA-BIOCAT1 (pRPA-BCAT23), RPA-BIOCAT1 (pRPA-BCAT24);

- C, E, G represent the supernatants obtained respectively from these same strains;

- H represents the pellet obtained from RPA-BIOCAT1 (pRPA-BCAT24);

- J, L, O represent the crude fractions obtained respectively from the strains G2081 (pRPA-BCAT14), G2081 (pRPA-BCAT23), G2081 (pRPA-BCAT24);

- K, N, P represent the supernatants obtained respectively from these same strains;

- Q represents the pellet obtained from G2081 (pRPA-BCAT24).

This experiment shows that the *P. putida* strains express large quantities of the three soluble nitrilase polypeptides and that only the nitrilase polypeptide at 44 kDa is overexpressed by the

5 *A. faecalis* strain. The assays of activity of these cultures, carried out according to the procedure described in Example 4 show that the strains G2081 (pRPA-BCAT23), G2081 (pRPA-BCAT24), and RPA-BIOCAT1 (pRPA-BCAT24) have a nitrilase activity on HMTBN.

10 **Example 7: Expression of the nitrilase in *Corynebacterium glutamicum*.**

The expression of the nitrilase was performed in the strain CGL1010 (= ATCC 14752) with the aid of the P *cspB* promoter (Peyret et al., 1993, Molecular

15 Microbiol. 9: 97-109). A 530 bp fragment containing the P *cspB* promoter was amplified from the plasmid pCGL815 (Peyret et al., cited above) with the aid of the following primers KS1 and KS2:

KS1: 5'-ACGCGTCGACCAGATCGTCAAGTTGTGG-3'

20 KS2: 5'-CATAGAGGCGAAGGCTCCTTG-3'

After digestion with *Sal*I, the 530 bp fragment amplified was cloned into the plasmid pBSK (Statagene, La Jolla, USA) opened by *Sal*I and *Eco*RV to give the plasmid pCGL1084. An *Eco*NI/*Nde*I adaptor was

25 prepared by hybridizing the following two

oligonucleotides KS8 and KS9:

KS8: 5'-TCAAGGAGCCTTCGCCTCA-3'

KS9: 5'-TATGAGGCGAAGGCTCCTTG-3'

The nitrilase gene was extracted from the
5 plasmid pRPA-BCAT6 in the form of a 1.1 kb NdeI-XbaI
fragment. It was introduced into pCGL1084 opened with
EcoNI and XbaI using the EcoNI/NdeI adaptor described
above, to give the plasmid pCGL1086. The SalI-BamHI
fragment of pCGL1086 containing P_{cspB}::nitB was then
10 cloned into the vector pCGL482 (Peyret *et al.*, cited
above) at the SalI and BamHI sites, giving the plasmid
pCGL1087. The plasmid pCGL1087 (Figure 10) is therefore
a shuttle vector based on pBI1 (Santamaria *et al.*,
1984, J. Gen. Microbiol. 130: 2237-2246) containing the
15 replication origin of pACYC 184 recognized in *E. coli*,
a gene conferring chloramphenicol (Cm) resistance and
the P_{cspB}::nitB fusion.

The plasmids pCGL1087 and pCGL482 were
introduced by electroporation into CGL1010 as described
20 by Bonnamy *et al.*, 1990 (FEMS Microbiol. Lett. 66: 263-
270). After culturing for 20 hours at 30°C in a 3.7%
Brain Heart Infusion medium (Difco Laboratories,
Detroit, USA) supplemented with 5 mg/l of
chloramphenicol, assays of nitrilase activities were
25 carried out on culture samples as described in

Example 4. The results show that the strain CGL1010 (pCGL1087) has a nitrilase activity whereas the strain CGL1010 (pCGL482) has none.

Example 8: Expression of the nitrilase in *Streptomyces lividans*.

The expression of the nitrilase was performed in the *S. lividans* TK24 strain (Hopwood et al., 1985, Genetic Manipulation of *Streptomyces*; A laboratory Manual, The John Innes Foundation, Norwich) with the aid of the plasmid pIJ6021 (Takano et al., 1995, Gene 166: 133-137) using the *PtipA* promoter (Holmes et al., 1993, EMBO J. 12: 3183-3191).

The plasmid pUC19 (Yanisch-Perron et al., 1985, Gene 33: 103-119) was modified by deleting the 140 bp *TfiI* fragment by *TfiI* digestion, treatment with Klenow and self-ligation of the vector. The plasmid obtained was opened with *EcoRI* and *BamHI* so as to clone the 1.15 kb *EcoRI*-*BamHI* fragment of pRPA-BCAT3 containing the *nitB* gene. The plasmid pOS48.4 thus obtained has a unique *TfiI* site situated between the *nitB* gene and the *BamHI* site. This site was used to insert a 2.3 kb Ω hyg fragment extracted from the plasmid pHP45 Ω hyg (Blondelet-Rouault et al., Gene, submitted) after digestion with *BamHI* and treatment with Klenow. The Ω hyg fragment contains the hygromycin phosphotransferase (*hyg*) gene of *Streptomyces hygroscopicus* (Zalacain et al., 1986, Nucl. Acids Res.,

14: 1565-1581) and confers hygromycin resistance on
S. lividans. The plasmid pOS48.5 thus obtained was used
to isolate a 3.45 kb NdeI-BamHI fragment containing the
nitrilase gene followed by the *hyg* gene, which was
5 cloned into the plasmid pIJ6021 (Takano et al., cited
above) opened with NdeI and BamHI. Given that the
plasmid pIJ6021 replicates only in the *Streptomyces*,
the ligation mixture was transformed in *S. lividans*
TK24 by conventional methods (Hopwood et al., cited
10 above) by selecting the clones resistant to 200 mg/l of
hygromycin (Boehringer Mannheim). The plasmid DNAs of
these clones were extracted by conventional methods
(Hopwood et al., cited above) and their restriction
patterns indeed corresponded to the expected construct
15 which was called pOS48.7 (Figure 11).

Two *S. lividans* strains containing pOS48.7 as
well as a clone containing the plasmid pIJ6021 were
cultured in 50 ml of TSB medium (Tryptic Soy Broth,
Difco) at 30°C for 72 h with a 5 mg/l kanamycin
20 selection, and then thiostrepton was added at the
concentration of 5 mg/l and the culture was continued
for another 18 h according to the conditions described
(Hopwood et al., cited above). The culture was
harvested and the activity assay, carried out under the
25 conditions described in Example 4, shows that the
S. lividans TK24 strain (pOS48.7) expresses a nitrilase
activity contrary to the TK24 strain (pIJ6021).

Example 9: Hydrolysis of HMTBN with other nitrilases

The primary sequence of the *Comamonas testosteroni* sp., nitrilase described in Levy-Schil et al., 1995 (cited above) exhibits 31% identity with the primary sequence of the nitrilase described in this invention. The recombinant strain of *E. coli* TGI (pXL2158, pXL2035), which expresses the *C. testosteroni* nitrilase, was cultured under the conditions described by Levy-Schil et al. (cited above) and a cellular pellet was incubated in 100 mM phosphate buffer at pH 7 with 50 mM HMTBN, at 30°C. The measured activity was 2.3 kg/h.kg CS. Like the nitrilase of the invention, the *C. testosteroni* nitrilase is capable of hydrolysing HMTBN.

Furthermore, the data presented in Example 4 with the plasmids pBCAT12 and pBCAT13 show that the Asn279→Asp279 substitution on the *A. faecalis* ATCC8750 nitrilase makes it possible to conserve the activity on HMTBN.

Example 10: Supply of the support

5 g of dry cells were added to 20 g of water adjusted to pH 7.0. The quantity of support (CELITE 545, Prolabo, France) indicated is then added and after a perfect homogenization, the suspension is cross-linked by addition of 15% glutaraldehyde based on the total dry mass followed by 10% polyethyleneimine (SEDIPUR, BASF, Germany). The suspension is stirred for

1 hour at room temperature.

The suspension is then flocculated by addition of 0.001% of an anionic flocculating agent, Superfloc A100. The cross-linked mass is recovered by
5 filtration.

This mass is then again cross-linked by addition of 10% polyazetidine (KYMENE 557, Hercules, USA) based on the total dry mass. The mass, still wet, is then extruded through an orifice of 0.5 mm in
10 diameter and dried.

The activity of the particles obtained is then determined according to a procedure described in Patent WO 96/09403.

	g of support	activity (in%)
15	0	100
	2.5 g	215
	5 g	250

The addition of a support for the cells considerably enhances the activity.

20 The experiment was repeated by replacing the CELITE with wheat gluten (Roquette, France) partially soluble in water or gelatin (SBI, France) completely soluble in water.

Support	% activity
0	100
5 g of gluten	160
5 g of gelatin	nd*

5 * The cellular suspension does not flocculate and is not filterable.

 This example shows that the gluten may be replaced by CELITE. On the other hand, if gelatin (completely soluble) is added, the forming of the catalyst is impossible by the technique described above (extrusion).

Example 11

 10 g of *Escherichia coli* BIOCAT171 of Example 4 prepared by aerobic culture in an Lb medium were mixed with 10 g of CLARCEL 78 (CECA, France) in 500 g of 100 mM phosphate buffer pH 7.0. After homogenization, 6 g of 25% glutaraldehyde were added and the suspension was stirred for 15 minutes at room temperature. 2 g of polyethylenimine were then added as well as 6 g of 25% glutaraldehyde. The whole was stirred for one hour at room temperature. The mixture was flocculated by the additional of 5 ml of a solution of SUPERFLOC A100 at 0.2% (CYTEC, France). The mass was filtered and the paste obtained was mixed with 16 g of polyazetidine at 12.5% (KYMENE 557, Hercules) and then

extruded through an orifice of 0.5 mm in diameter. The vermicelli obtained were dried at 35°C in an oven and then immersed for 30 minutes in a 1% NaBH₄ bath prepared in a 50 mM borate buffer pH 10.0. The biocatalyst was
5 then washed with distilled water. The catalyst is stored at 5°C or at room temperature in 500 mM phosphate buffer pH 8.0.

A thermostated column with an inner diameter of 3 cm and a height of 45 cm was filled with 100 g of
10 biocatalyst. This column is connected to a pump via a recirculation loop. The total volume of the reactor is 430 ml. The loop is filled with a 25% solution of ammonium salt of hydroxymethylthiobutyric acid. The solution is fed through the top of the column downwards
15 at an hourly flow rate of 20 l/h. The water which circulates in the jacket of the column and in the heat exchanger makes it possible to maintain the temperature at 35°C. Demineralized water is added to the loop at a flow rate of 80 g/h. The 2-hydroxy-4-methylthio-
20 butyronitrile at 20 g/h. The excess volume of the reaction medium is evaporated through the bottom of the column such that the volume of the loop remains constant. Thus, a continuous flow is obtained with a nitrile conversion rate of 95%. Furthermore, the
25 concentration of the aqueous solution of ammonium salt of 2-hydroxy-4-methylthiobutyric acid obtained at the outlet of the reactor is 25%.

Example 12

The electrodialyser used consists of a stack of 9 cells with an active surface area of 2 dm², each of them composed of 2 compartments designed as follows:

5 - salt/acid compartment: delimited on the cathode side by a cation-exchange membrane Neosepta CMB from Tokuyama Soda, and on the anode side by the cationic face of an Aqualytic bipolar membrane,

 - base compartment: delimited on the anode
10 side by the cationic membrane, and on the cathode side by the anionic face of the bipolar membrane.

The anode consists of platinized titanium.
The cathode is made of stainless steel.

The electrolyte consists of an aqueous sodium
15 sulphate solution having a conductivity of 100 mS/cm at 40°C. The circulation rate at the electrodes is 2 x 100 l/h. The volume is 5 l.

The "base" compartment is initially filled with 5 litres of a 1% ammonium sulphate solution.

20 The "salt/acid" compartment is initially filled with 5 litres of a solution at 1.44 mol/l of the ammonium salt of 2-hydroxy-4-(methylthio)butanoic acid.

The recirculation rate of the solutions is initially fixed at 130 l/h for the salt/acid
25 compartment and at 190 l/h for the base compartment.

The electrodialysis is performed in a batch mode (operation with recirculation), at a mean temperature of 40°C. The intensity is set at 9A, that

is to say a current density of 0.45 kA/m^2 .

After 155 minutes of operation, the conductivity of the salt/acid compartment passed from 59 to 7.8 mS/cm .

5 The salt/acid compartment contains 1.35 mol/l of 2-hydroxy-4-(methylthio)butanoic acid, at 100% in acid form.

The faradic yield is estimated at 71% and the energy consumption at 0.53 kWh per kg of acid formed.

10 **Example 13**

The electrodialyser used consists of a stack of 8 cells with a configuration identical to that of Example 12.

15 The "base" compartment is initially filled with 5.27 litres of a 1% ammonium sulphate solution.

The "salt/acid" compartment is initially filled with 4.85 litres of a solution at 1.39 mol/l of the ammonium salt of 2-hydroxy-4-(methylthio)butanoic acid.

20 The recirculation rate of the solutions is initially fixed at 60 l/h for the salt/acid compartment and at 150 l/h for the base compartment.

The electrodialysis is performed in a batch mode (operation with recirculation), at a mean
25 temperature of 40°C . The intensity is set at 9A , that is to say a current density of 0.45 kA/m^2 .

After 172 minutes of operation, the

conductivity of the salt/acid compartment passed from 59 to 9.5 mS/cm.

The final volume of the salt/acid compartment is 4.67 litres.

5 The composition is the following:

2-hydroxy-4-(methylthio)butanoic acid: 1.24 mol/l

ammonium 2-hydroxy-4-(methylthio)butanoate: 0.148 mol/l

The conversion rate is 86%. The final product
10 is 89% in acid form.

The faradic yield is 74%. The energy consumption is estimated at 0.58 kWh per kg of acid formed.

Example 14

15 A device similar to that of Example 13 is used.

The "base" compartment is initially filled with 5.46 litres of a 1% ammonium sulphate solution.

The "salt/acid" compartment is initially
20 filled with 5.23 litres of a solution at 1.24 mol/l of the ammonium salt of 2-hydroxy-4-(methylthio)butanoic acid.

The recirculation rate of the solutions is initially fixed at 90 l/h for the salt/acid compartment
25 and at 150 l/h for the base compartment.

The electrodialysis is performed in a batch mode (operation with recirculation), at a mean temperature of 40°C. The intensity is set at 14 A, that is to say a current density of 0.7 kA/m².

5 After 105 minutes of operation, the conductivity of the salt/acid compartment passed from 57.6 to 9.8 mS/cm.

The final composition of the salt/acid compartment is the following:

10 2-hydroxy-4-(methylthio)butanoic acid: 1.28 mol/l

ammonium 2-hydroxy-4-(methylthio)butanoate: 0.14 mol/l

The product is therefore 90% in acid form.

Example 15

A device similar to that of Example 13 is
15 used.

The trial is carried out with the content of the base compartment obtained at the end of Example 14.

The "salt/acid" compartment is initially filled with 5.2 litres of a solution at 1.44 mol/l of
20 the ammonium salt of 2-hydroxy-4-(methylthio)butanoic acid.

The recirculation rate of the solutions is initially fixed at 50 l/h for the salt/acid compartment and at 150 l/h for the base compartment.

The electrodialysis is performed in a batch mode (operation with recirculation), at a mean temperature of 42°C. The intensity is set at 19 A, that is to say a current density of 0.95 kA/m².

5 After 53 minutes of operation, the conductivity of the salt/acid compartment passed from 59 to 27 mS/cm.

The final volume of the salt/acid compartment is 4.85 litres.

10 The composition is the following:

2-hydroxy-4-(methylthio)butanoic acid: 0.87 mol/l

ammonium 2-hydroxy-4-(methylthio)butanoate: 0.56 mol/l

The conversion rate is 56%. The final product
15 is 61% in acid form.

The faradic yield is 83%. The energy consumption is estimated at 0.7 kWh per kg of acid formed.

Example 16

20 200.1 g of HMTBS solution at about 1.5 M are concentrated batchwise in a rotary evaporator. The temperature of the bath is 45+/-5°C. The pressure is regulated at around 2.5×10^3 Pa (25 mbar). The temperature in the distiller varies from 25°C at the
25 start of distillation up to 40°C at the end. After

3 hours, 41.9 g of a yellow viscous medium are recovered whose characteristics are the following:

Viscosity at 25°C (mm ² ·s ⁻¹)	1150
% HMTBA (Br/BrO ₃ ⁻)	83.3

5

After adjusting the titre (dilution with H₂O), a product is obtained whose characteristics are the following:

Viscosity at 25°C (mm ² ·s ⁻¹)	396
% HMTBA (Br/BrO ₃ ⁻)	79.5

10

The following mass distribution is obtained by potentiometry:

% HMTBS	81.6
% HMTBA	6.0

15

The ratio of the areas monomer or dimers/(monomer+dimers) is measured by HPLC

monomer/(monomer+dimers) 100

20

dimers/(monomer+dimers) (*) 0

(*) no dimers are detected

Example 17

200.0 g of HMTBS solution at about 1.5 M are concentrated batchwise in a rotary evaporator. The temperature of the bath is 121/-5°C. The pressure is regulated at around 9.5×10^4 Pa. The temperature in the distiller varies from 100°C at the start of distillation up to 113°C at the end. After 3 hours, 41.5 g of a brown viscous medium are recovered whose characteristics are the following:

10

Viscosity at 25°C ($\text{mm}^2 \cdot \text{s}^{-1}$)	-
% HMTBA (Br/BrO3-)	90.7

After adjusting the titre (dilution with H_2O), a product is obtained whose characteristics are the following:

15

Viscosity at 25°C ($\text{mm}^2 \cdot \text{s}^{-1}$)	117
% HMTBA (Br/BrO3-)	78.8

20

The following mass distribution is obtained by potentiometry:

% HMTBS	52.2
% HMTBA	28.7

The ratio of the areas monomer or

dimers/(dimers+monomer) is measured by HPLC

monomer/(monomer+dimers) 95.0

dimers/(monomer+dimers) 5.0

Example 18

5 40.0 g of H₂O are added to 100.0 g of the
medium described in Example No. 17. The medium is
extracted with 75.0 g of isopropyl ether. After
separation of the phases, 25.6 g of HMTBA, whose
characteristics are the following, are recovered in the
10 organic phase after evaporation:

15

Determination	
Monomer/(monomer+dimers) (% area)	97.0
Dimers/(monomer+dimers) (% area)	3.0
Viscosity at 25°C (mm ² ·s ⁻¹)	55

Example 19: Ageing of the solutions described in the preceding examples

	Example No. 16	Example No. 17	Example No. 18
Duration of storage at 25°C	40 d	40 d	100 d
5 Viscosity ($\text{mm}^2 \cdot \text{s}^{-1}$)	387	116	66
Monomer/ (monomer+dimer)	100.0	95.0	82
Dimers/ (monomer+dimers)	0.0	5.0	18

The solutions of Examples 16 and 17 did not vary in their content of dimers after 40 days of storage.

10

CLAIMS

1. Process for the preparation of
2-hydroxy-4-methylthiobutyric acid and/or of the
ammonium salt of 2-hydroxy-4-methylthio-butyric acid by
5 enzymatic hydrolysis of 2-hydroxy-4-methylthio-
butyronitrile, characterized in that:

a) in a first stage, a biological material
having a nitrilase activity is prepared,

b) in a second stage, it is immobilized,

10 c) in a third stage, the 2-hydroxy-4-
methylthiobutyronitrile is exposed to the biological
material thus immobilized, in order to obtain the
ammonium salt of 2-hydroxy-4-methylthiobutyric acid,

d) in a fourth stage, optionally, the salt
15 obtained in stage c) is converted to the corresponding
acid, and

e) in a fifth stage, the product obtained in
stage c) or d) is concentrated.

2. Process according to claim 1,
20 characterized in that the nitrilase activity is
obtained from an *Alcaligenes*, preferably *faecalis*,
nitrilase.

3. Process according to claim 1 or claim 2,
characterized in that the genetic information encoding
25 a nitrilase is used which is expressed in a host
microorganism.

4. Process according to any one of claims 1
to 3, characterized in that the host microorganism is



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chosen from *Escherichia coli* or a member of the genus *Bacillus*, *Corynebacterium*, *Streptomyces*, *Saccharomyces*, *Kluyveromyces*, *Penicillium* and *Aspergillus*.

5. Process according to claim 1,
5 characterized in that the nitrilase activity is obtained from the nitrilase encoded by the gene cloned into the plasmid pRPA-BCAT3, deposited at CBS under the number CBS 998-96.

6. Process according to any one of claims 1
10 to 5, characterized in that the nitrilase is coexpressed with a chaperon protein.

7. Process according to claim 5,
characterized in that the chaperon protein is GroESL in *E. coli*.

15 8. Process according to any one of the preceding claims, characterized in that the biological material of the stage a) is immobilized on a solid support.

9. Process according to claim 8,
20 characterized in that the solid support has a particle size of between 1 μm and 3 mm, preferably 10 μm and 2 mm, and in that it is added in an amount of 0.01 to 500%, preferably 10 to 200% by weight of the biological material.

25 10. Process according to claim 9, [lacuna] in that the solid support is chosen from:

- ion-exchange resins,
- alumina,

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- synthetic silicas or diatomaceous earth and silica gels,
- zeolites,
- charcoals,
- partially water-soluble proteins, and
- polysaccharides.

11. Process according to claim 10, characterized in that the solid support is gluten.

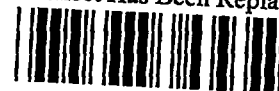
10 12. Process according to claim 8, characterized in that one or more chemical agents are used to cross-link and/or insolubilize the biological material and the support.

13. Process according to claim 12, characterized in that the chemical agents are chosen
15 from:

- polyazetidine polymers,
- polyethyleneimine polymers,
- polyamide polymers,
- isocyanate polymers,
- 20 - alginate gels,
- k-carrageenan gels,
- amines,
- aldehydes,
- carboxylic acids, and
- 25 - isocyanates.

14. Process according to claims 10 to 13, characterized in that the biological material and the support are mixed in the presence of chemical agents in

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order to obtain a paste which is extruded and then dried.

15. Process according to claims 10 to 13, characterized in that the biological material and the chemical agents are mixed and then deposited on the support in the form of a thin layer.

16. Process according to any one of the preceding claims, characterized in that a mixture of HMTBS and HMTBA containing at least 60% HMTBA, and preferably at least 80% HMTBA, the balance being HMTBS, is obtained by electrolytic dissociation of the corresponding ammonium salt.

17. Process according to claim 16, characterized in that the dissociation is carried out in an electrodialyser with bipolar membranes having three compartments.

18. Process according to claim 16, characterized in that the dissociation is performed in an electrodialyser with bipolar membranes having two compartments.

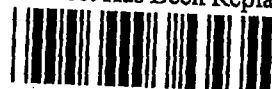
19. Process according to any one of claims 1 to 15, characterized in that a mixture of HMTBS and HMTBA is obtained by heating an ammonium salt solution.

20. Process according to claims 16 to 19, characterized in that the liberated ammonium hydroxide is distilled, concentrated and recycled in the HCN synthesis.

21. Aqueous solution obtained according to

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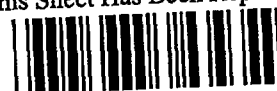
claims 16 to 19, consisting of a mixture of HMTBA and HMTBS whose HMTBA/(HMTBA+HMTBS) weight ratio is 5 to 99.9% and preferably 10 to 50%.

22. Plant for carrying out the process
5 according to the invention comprising:

- one or more reactors (3, 4) packed with immobilized enzymes having a nitrilase activity, or with immobilized host microorganisms expressing an enzyme with nitrilase activity,
- 10 - optionally, one or more electrodialysis means (9),
- means for concentration (6, 12) of the final product.

23. Plasmid pRPA-BCAT3, deposited at CBS
15 under the number CBS 998-96.

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