The biotransformation of carboxylic acids in the presence of a microorganism

The present invention relates to a process for the biotransformation of carboxylic acids in the presence of a microorganism. Biotransformation means the selective chemical conversion of substances of defined purity (precursors) to defined final products, this conversion being catalyzed by microorganisms, animal or vegetable cell cultures or isolated enzymes.

The biotransformation of carboxylic acids is carried out in order, for example, to achieve regio- or enantioselective reactions such as hydroxylations and epoxidations which are very complicated, if possible at all, by chemical synthesis.

DE-A 39 10 024 describes the regioselective hydroxylation of 2-phenoxypropionic acid to 2-(4-hydroxyphenoxy)propionic acid using various microorganisms. It is possible to hydroxylate both racemates and enantiomerically pure compounds. The advantage of this process is that no hydroxylated byproducts are formed.

However, this process is still in need of optimization in terms of economics. In particular, the conversion is no longer complete at high precursor concentrations above 50 g/l, or else the fermentation time is greatly increased, which has adverse effects on the economics of the process.

It is an object of the present invention to provide an improved process for the biotransformation of carboxylic acids in the presence of microorganisms which does not have the disadvantages described above.

We have found that this object is achieved by a process for the biotransformation of carboxylic acids in the presence of a microorganism, wherein a concentration, which is optimal for the biotransformation, of undissociated carboxylic acid is adjusted throughout the biotransformation by controlling the pH.

The pH often changes very greatly in the course of a biotransformation. This is influenced by a number of factors, such as metabolytes produced, nutrients consumed, and changes in the precursor to product ratio.

The pH plays a crucial part in the biotransformation of carboxylic acids because its value fixes the proportions of the undissociated and dissociated forms of the carboxylic acid. These

proportions can easily be found with the aid of the known buffer equation when the pKa of the carboxylic acid is known.

An increase in the pH very generally leads to an increase in the proportion of dissociated acid, while a reduction in the pH increases the proportion of undissociated acid.

The undissociated form of the carboxylic acid is the form which is taken up by microorganisms and, where appropriate, transformed.

It is therefore desirable to achieve the maximum proportion of undissociated carboxylic acid by controlling the pH because this sets up a high transmembrane concentration gradient which leads to a high rate of diffusion of the precursor into the cell. However, the proportion of undissociated form must not be so high that it has toxic or growth-inhibiting effects on the relevant microorganism.

The course of a biotransformation comprises various stages which, however, mutually overlap and cannot be delimited absolutely unambiguously.

The first phase is determined by the growth of the microorganisms. For an economic process, it is desirable for there to be a rapid build-up of productive biomass in the presence of the precursor during this. As a rule, 25–50% of the biomass present at the end of the biotransformation is formed in this phase. The first phase generally takes up 10–30% of the total biotransformation time.

The critical factors in this first phase are the osmolarity of the medium and the concentration of the undissociated precursor. High values of one or both of the parameters inhibit the growth of the microorganisms or are toxic for them.

The pH in this first phase of the biotransformation is therefore advantageously controlled so that the proportion of the undissociated form of the precursor is from 1 to 30, preferably from 3 to 20, % of the growth-inhibiting concentration.

The growth-inhibiting concentration can easily be determined for a particular carboxylic acid and a given microorganism by simple preliminary tests familiar to the skilled worker. In addition, the pH to be adjusted can easily be calculated from knowledge of the pKa of the carboxylic acid and using the buffer equation.

In the second phase of the biotransformation most of the precursor is converted with high productivity. To ensure a high space-time yield, the concentration of the undissociated form of the precursor is adjusted so that it is 5–65, preferably 15–40, % of the growth-inhibiting concentration for a given organism.

In the third phase of the biotransformation, no further precursor is metered in and the precursor present in the medium is converted as completely as possible into product. As a rule, the pH is reduced in this phase, which results in the concentration of the undissociated form of the precursor remaining for as long as possible in the optimal range of the second phase even when the precursor concentration falls. This results in the diffusion rate, which limits the productivity, staying at a sufficiently high level.

This phase generally takes from 10 to 30% of the total biotransformation time.

It is possible by this procedure to achieve in the case of the hydroxylation of 2–phenoxypropionic acid to 2–(4–hydroxyphenoxy)propionic acid complete conversions (≥98%) at the end of the biotransformation within 10 days with product concentrations of >100 g/l.

The process according to the invention is suitable for the hydroxylation and epoxidation of carboxylic acids by biotransformation. Aliphatic and aromatic, preferably cyclic aliphatic and aromatic carboxylic acids are suitable.

The hydroxylation of carboxylic acids which have an aromatic radical (aromatic carboxylic acids) is particularly preferred. The regioselective hydroxylation of the aromatic radical takes place particularly well using the process according to the invention. The process according to the invention can be applied to the compounds described in DE–A 39 10 024, DE–A 41 34 774, DE–A 41 34 775, DE–A 41 42 943, DE–A 42 20 241 and DE–A 42 32 522.

Particularly suitable microorganisms for the process according to the invention are fungi and bacteria. Examples of suitable microorganisms are described in DE 39 10 024 or can easily be found by the processes described therein.

Particularly suitable microorganisms for the conversion of 2-phenoxypropionic acid into 2-(4-hydroxyphenoxy)propionic acid are those of the Beauveria genus.

The biotransformation is, as a rule, carried out by cultivating microorganisms in a nutrient medium which can, if required, already contain the precursor and, after conversion is as substantial as possible, isolating the product from this microorganism culture broth and purifying it where appropriate.

However, the precursor can also be added only during the course of the biotransformation, or mixed processes are applied, in which precursor is initially present and is added continuously or intermittently.

Addition during the biotransformation is particularly preferred because, in this case, it is unnecessary for the complete amount of precursor to be present at the outset, which often means that there is a great osmotic stress on the microorganisms.

Furthermore, the addition of a source of nutrients, such as of carbon or nitrogen or of carbon and nitrogen, during the biotransformation is also a preferred embodiment.

Particularly suitable microorganisms for the process according to the invention are those specially adapted to the osmotic conditions of the biotransformation (high osmolarity).

Organisms of this type can be obtained by classical mutagenesis by means of radiation or chemical agents and subsequent selection for growth in the presence of large amounts of precursor/product.

It is additionally possible to use transposable genetic elements for the mutagenesis.

However, organisms of this type can also be selected in continuous culture from a population of less adapted individuals by adding increasing concentrations of precursor/product in an appropriate manner to the medium flowing in.

The invention is illustrated further by the following examples.

Examples 1 to 4 describe a biotransformation without the pH control according to the invention, and Examples 5 and 6 with pH control.

Example 1

Preparation of 2-(4-hydroxyphenoxy)propionic acid without pH control and metering in of further sources of nutrients

The procedure described in this Example for the main culture for the preparation of 2-(4-hydroxyphenoxy)propionic acid by fermentation corresponds to the procedure described in DE-A 39 10 024.

Nutrient media A, B and C were prepared for the precultures, and nutrient medium D was prepared for the main culture.

		Medium A	Medium B	Medium C	Medium D
Glucose	g/l	20	20	40	100
2-Phenoxypropionic acid	g/l	50	65	85	45
Yeast extract	g/l	10	10	10	3
Urea	g/l	0	0	0	2.1
$MgSO_4 * 7 H_2O$	g/l	2	2	2	2
$CaCl_2 * 2 H_2O$	g/l	1	1	1	1
KH_2PO_4	g/l		0.75	0.75	0.75
K_2HPO_4	g/l	1.8	1.8	1.8	1.8
Trace element solution	g/l	50	50	50	50
Antifoam P2000	g/l	1	1	1	1

Trace element solution

2 M H_2SO_4	1	ml/l
Titriplex®	2000	mg/l
$FeSO_4 * 7 H_2O$	600	mg/l
$ZnSO_4 * 7 H_2O$	200	mg/l
$MnSO_4 * H_2O$	150	mg/l
H_3BO_3	30	mg/l
$CoCl_2 * 6 H_2O$	20	mg/l
$CuCl_2 * 2 H_2O$	40	mg/l
$NiCl_2 * 2 H_2O$	40	mg/l
$Na_2MoO_4 * 2 H_2O$	5	mg/l

2-Phenoxypropionic acid was employed in the form of a 50% strength aqueous solution of the Na salt.

The urea employed in the main culture was sterilized by filtration. Glucose and phosphates were autoclaved separately, the other constituents together. After the various fractions had been combined, the pH was adjusted to 6.8 with NaOH.

Beauveria bassiana (CMI No. 12942) was obtained by N-methyl-N'-nitrosoguanidine [sic] mutagenesis in the presence of 100 g/l of 2-phenoxypropionic acid at pH 6.8 in liquid culture. A corresponding method for preparing mutants using N-methyl-N'-nitrosoguanidine is to be found in Biochem. Biophys. Res. Commun. 18, (1965) 788. The selection for growth in the presence of high precursor concentrations was carried out by 5 passages in submerged cultures. The strain obtained in this way is called Lu 700 and is used for this and the following Examples.

The strain was cultivated on agar plates as working cell bank for preparing the inoculum. The agar plates were prepared by adding agar in a concentration of 18 g/l of medium to medium A. Transfer to new plates took place every 14 days. 1 to 3-week old agar plates were used for the precultures.

The preculture was prepared by introducing 100 ml of sterile medium A into each of four sterile 500 ml Erlenmeyer flasks. The flasks were inoculated with a small piece of mycelium of the strain Lu 700 from an agar plate and shaken at 28°C and 250 rpm for 2 days. This was used to inoculate a fermenter containing 9 l of sterile medium B and incubated at 800 rpm, 28°C and an aeration rate of 1 VVM for one day. 0.45 l of this culture was used to

inoculate a fermenter containing 9 l of medium C and likewise incubated at 800 rpm, 28°C and an aeration rate of 1 VVM for one day. This third culture was used to inoculate the main cultures of this Example and the following Examples.

To do this, a fermenter containing 9 l of medium D was inoculated with 0.9 l of the third culture and incubated under the same operating conditions as the previous fermenters for five days. The pH was not controlled. The change in pH during the fermentation was recorded. At the end of the fermentation, a sample was removed and the glucose concentration was determined enzymatically using the Boehringer Mannheim test kit No. 716 251, and the conversion was determined by gas chromatography (GC) by the method described in DE-A 39 19 024. The standards used for GC analysis calibration were authentic samples of 2-phenoxypropionic acid and 2-(4-hydroxyphenoxy)propionic acid.

The molar conversion was found from the concentrations of 2-phenoxypropionic acid and 2-(4-hydroxyphenoxy)propionic acid determined by gas chromatography and using the relevant molecular weights. The amount of product formed per fermenter at a particular time was determined from the amount of precursor employed and the molar conversion.

The following results were obtained after 5 days:

Time (days)	1	2	3	4	5
pH	6.9	5.7	5.0	4.8	4.7
Molar conversion (%)					98
Glucose (g/l)					0
2-(4-Hydroxyphenoxy)propionic acid (g/l)					52.5
Amount of 2-(4-hydroxyphenoxy)propionic acid (g) per fermenter					483

Example 2

Preparation of 2-(4-hydroxyphenoxy)propionic acid with a higher precursor concentration

The sequence of precultures described in Example 1 was carried out with the strain Lu 700, and a fermenter containing 9 l of medium D was inoculated with 0.9 l of the 3rd culture. However, in this case, medium D contained 100 g/l of phenoxypropionic acid. The operating conditions detailed in Example 1 were set up. The pH was not controlled, but the change with time was recorded.

A sample was taken after 5 and 7 days and analyzed as described in Example 1.

Time (days)	1	2	3	4	5	6	7
pH	7.3	7.2	7.1	6.8	6.3	6.4	6.5
Molar conversion (%)					19		25
Glucose (g/l)					15		0
2-(4-Hydroxy-phenoxy)propionic acid (g/l)					22.3		29.8
Amount of 2-(4-hydroxyphenoxy)propionic acid (g) per fermenter					187		247

Example 3

Preparation of 2-(4-hydroxyphenoxy)propionic acid with addition of the C source and increased precursor concentration

The sequence of precultures described in Example 1 was carried out with the strain Lu 700, and a fermenter containing 9 l of medium D was inoculated with 0.9 l of the 3rd preculture, the glucose concentration in medium D having been changed to 60 g/l and the 2-phenoxypropionic acid concentration having been changed to 90 g/l. The fermenter was operated with the values described in Example 1 for the temperature, aeration rate and stirrer speed. As in Example 2, the pH was not controlled but the change with time was recorded. From day 2 to day 3, 250 g of glucose/fermenter/day were added in the form of a 55% strength solution. A sample was taken after 5 and 7 days and analyzed as in Example 1. The following results were obtained:

Time (days)	1	2	3	4	5	6	7
pH	7.2	6.9	6.6	5.7	5.7	5.7	5.5
Molar conversion (%)					28.9		28.9
Glucose (g/l)					34.5		35
2-(4-Hydroxyphenoxy)propionic acid (g/l)					28		29.2
Amount of 2-(4-hydroxyphenoxy)propionic acid (g) per fermenter					257		257

Example 4

Preparation of 2-(4-hydroxyphenoxy)propionic acid with addition of a C and N source and increased precursor concentration

The sequence of precultures described in Example 1 was carried out with the strain Lu 700, and a fermenter containing 9 l of medium D was inoculated with 0.9 l of the 3rd preculture, the glucose concentration in medium D having been changed to 60 g/l and the 2-phenoxypropionic acid concentration having been changed to 90 g/l. The operating conditions correspond to those described in Example 1. During the fermentation, the following solutions were metered in continuously in accordance with the following criteria:

	Start	End	Rate (based on 100% substance)
Glucose 55% strength (W/W)	Day 2	Day 9	340 g/fermenter/day
Urea 6.75% strength (W/W)	Day 2	Day 9	6.75 g/fermenter/day

The pH was kept constant at 6.5 or above with 5 M NaOH by an appropriate control system, and the actual value each day was recorded.

Samples were taken from the fermenter after 5, 7 and 9 days and analyzed as described in Example 1. The following results were obtained:

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Time (days)	1	2	3	4	5	6	7	8	9
pH	6.9	6.6	6.5	6.5	6.5	6.5	6.5	6.5	6.5
Molar conversion (%)					36.1		59.5		67
Glucose (g/l)					1		0		0
2-(4-Hydroxy-phenoxy)propionic acid (g/l)					36.3		49		56.7
Amount of 2-(4-hydroxy-phenoxy)propionic acid (g) per fermenter					320		528		595

Example 5

Preparation of 2-(4-hydroxyphenoxy)propionic acid with pH control

The sequence of precultures described in Example 1 was carried out with the strain Lu 700, and a fermenter containing 9 l of medium D was inoculated with 0.9 l of the 3rd preculture, the glucose concentration in medium D having been changed to 60 g/l and the 2-phenoxypropionic acid concentration having been changed to 90 g/l. The fermenter was operated under the conditions described in Example 1.

During the fermentation, the following solutions were metered in continuously in accordance with the following criteria.

	Start	End	Rate (based on 100% substance)
Glucose 55% strength (W/W)	Day 2	Day 7	320 g/fermenter/day
Urea 6.75% strength (W/W)	Day 2	Day 7	6.75 g/fermenter/day

The change in pH was controlled each day with 5 M NaOH in accordance with the values indicated in the following Table. A sample was taken after 5 and 7 days and analyzed as described in Example 1. The following results were obtained:

Time (days)	1	2	3	4	5	6	7
pH	7.5	7.1	6.5	6.1	6.0	5.8	5.5
Molar conversion (%)					75		99
Glucose (g/l)					5		10
2-(4-Hydroxyphenoxy)propionic acid (g/l)					73		96
Amount of 2-(4-hydroxyphenoxy)propionic acid (g) per fermenter					665		880

Example 6

Preparation of 2-(4-hydroxyphenoxy)propionic acid with pH control and metering in of precursor

The sequence of precultures described in Example 1 was carried out with the strain Lu 700, and a fermenter containing 9 l of medium D was inoculated with 0.9 l of the 3rd preculture, the glucose concentration in medium D having been changed to 60 g/l and the 2-phenoxypropionic acid concentration having been changed to 90 g/l. The operating conditions correspond to those described in Example 1. During the fermentation the following solutions were metered in continuously in accordance with the following criteria:

	Start	End	Rate (based on 100% substance)
Glucose 55% strength (W/W)	Day 2	Day 9	350 g/fermenter/day
Sodium 2-phenoxypropionate 50% strength (W/W)	Day 4	Day 6	90 g/fermenter/day
Urea 6.75% strength (W/W)	Day 2	Day 9	6.75 g/fermenter/day

The change in pH was controlled each day with 5 M NaOH or 2 M H_2SO_4 in accordance with the values listed in the following table of results.

Samples were taken after 5, 7 and 9 days and analyzed as described in Example 1:

Time (days)	1	2	3	4	5	6	7	8	9
pH	7.1	6.5	6.3	6.1	6.1	6.1	5.9	5.7	5.1
Molar conversion (%)					67		85		99
Glucose (g/l)					1		3		3
2-(4-Hydroxy-phenoxy)propionic acid (g/l)					70		97		103
Amount of 2-(4-hydroxy-phenoxy)propionic acid (g) per fermenter					658		1012		1178

We claim:

1. A process for the biotransformation of carboxylic acids in the presence of a microorganism, wherein a concentration, which is optimal for the biotransformation, of undissociated carboxylic acid is adjusted throughout the biotransformation by controlling the pH.

2. A process as claimed in claim 1, wherein an aromatic carboxylic acid is used for the biotransformation.

3. A process as claimed in claim 2, wherein the biotransformation is a hydroxylation of the aromatic nucleus of the carboxylic acid.

4. A process as claimed in claim 3, wherein the biotransformation is a para-hydroxylation of 2-phenoxypropionic acid.

5. A process as claimed in any of claims 1 to 4, wherein a source of nutrients is metered in during the biotransformation.

6. A process as claimed in any of claims 1 to 5, wherein a precursor is metered in during the biotransformation.

7. A process as claimed in any of claims 1 to 6, wherein microorganisms which have been specially adapted to the osmotic conditions of the biotransformation are used as microorganisms.

8. A process for preparing 2-(4-hydroxyphenoxy)propionic acid from 2-phenoxypropionic acid using a biotransformation as claimed in any of claims 1 to 7.

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The biotransformation of carboxylic acids in the presence of a microorganism

Abstract

A process for the biotransformation of carboxylic acids in the presence of a microorganism, wherein a concentration, which is optimal for the biotransformation, of undissociated carboxylic acid is adjusted throughout the biotransformation by controlling the pH.