

Process for preparing thiols

The invention relates to the preparation of thiols, in particular natural flavouring thiols, by bioconversion.

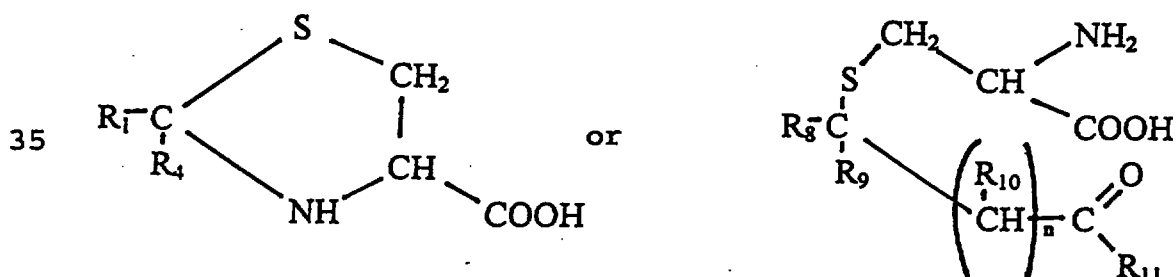
Thiols are among the volatile sulphur-containing substances which are considered to be responsible for the meaty note of various meats, for example, chicken, beef and pork, or which are components of the flavour of coffee.

Meat flavourings are generally produced by a thermal reaction between a reducing sugar and an amino acid in the presence of a sulphur-containing compound as the source of sulphur, for example cysteine, at a high temperature for a certain period of time, optionally in the presence of fat. Such a reaction provides a complex mixture of aromatic substances entering into the composition of the flavour of meat.

The object of the present invention is to provide natural thiols, obtained by bioconversion, capable of acting as flavouring ingredients for foods.

It is known, for example from US-A-5,182,194, that certain micro-organisms which cannot be used in food are able to provide a beta-C-S-lyase enzyme capable of producing thiols from cysteine S-complexes.

The process according to the invention is characterized in that a cysteine S-complex with the formula :



in which :

- R_1 is H, C_{1-7} linear or branched alkyl, phenyl, benzyl, 2- R_2 -2- R_3 -vinyl, 5- R_2 -furan-2-yl, 5- R_2 -thiofuran-2-yl,
- 5 R_2 and R_3 are similar or different and represent H or CH_3 , R_4 is H, or forms with R_1 a 2- R_5 -5- R_6 -tetrahydrofuran-3-ylidene, or 2- R_5 -5- R_6 -2,3-dihydrofuran-3-ylidene group, in which R_5 and R_6 are similar or different and represent H or CH_3 ,
- 10 or R_4 is a $CH(OH)R_7$ group and R_7 represents H, C_{1-4} -alkyl or phenyl, R_8 , R_9 and R_{10} are similar or different and represent H or C_{1-4} -alkyl, R_{11} represents H, C_{1-4} -alkyl, phenyl, C_{1-4} -alkoxy, or
- 15 C_{1-4} alkylamino and n is 0 or 1,

is put into contact with a micro-organism which can be used in food, having an enzymatic activity of the beta-C-S-lyase type.

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Within the context of the invention, an R_1 radical with C_{1-7} is preferably methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, n-hexyl or n-heptyl.

- 25 An R_8 to R_{10} radical is preferably H or methyl and R_{11} is preferably methyl, ethyl, phenyl, methoxy, ethoxy, methylamino or ethylamino.

- Under the action of beta-C-S-lyase, the cysteyle radical of
- 30 the complex compound is split in the beta position from the end carboxyl group leading to the corresponding thiol.

- When n is 0, the compound formed by the action of beta-C-S-lyase is then an alpha-mercapto aldehyde, an alpha-
- 35 mercaptoacetone, an alpha-mercaptoester or an alpha-mercaptoamide.

When $n = 1$, the compound formed by the action of beta-C-S-lyase is then a beta-mercapto aldehyde, a beta-mercaptoacetone, a beta-mercaptoester or a beta-mercaptoamide.

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The preferred compounds liberated by beta-C-S-lyase are furfuryl thiol (FFT), benzyl thiol, 5-methylfurfuryl thiol, 2-methylfuran-3-thiol, 2,5-dimethylfuran-3-thiol, 2-methyl-tetrahydrofuran-3-thiol and 3-methyl-2-butene-1-thiol.

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These compounds contribute significantly to the generation of flavours of the meat, chicken, beef and pork type, and also coffee as regards furanthiols and 3-methyl-2-butene-1-thiol.

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It has been found that the preceding preferred compounds can be partly metabolized into furfuryl methyl sulphide (Me-FFT), S-furfurylthio acetate (Ac-FFT) and bis-furfuryl disulphide (Di-FFT). These metabolites also possess a characteristic meaty note and may also contribute to the formation of flavour.

According to the invention, the micro-organisms involved are those which can be used in food and are capable of possessing enzymatic activity of the beta-C-S-lyase type.

These are preferably yeasts and filamentous fungi or moulds. Among the yeasts, reference may particularly be made to baker's yeast, *Saccharomyces cerevisiae*, but also, for example, brewer's yeast, and the yeasts *Candida versatilis*, *Debaromyces hansenii*, *Saccharomyces bayanus* and *Saccharomyces rouxii*. Among the filamentous fungi, reference may be made, for example, to *Aspergillus oryzae*, *Penicillium roqueforti* and *Penicillium camemberti*.

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Cysteine complexes able to act as a substrate in bioconversion according to the invention may be easily

obtained by reacting an aldehyde or a ketone of the formula $R_1\text{-CHO}$ or $R_1, R_4\text{-CO}$ or a ketone, ester or amide, alpha, beta-unsaturates of the formula $CR_8, R_9 = (CR_{10})_n \text{COR}_{11}$, in which R_1 to R_{11} have the preceding meanings, with cysteine in a water-alcohol medium at room temperature.

The incubation reaction with a micro-organism takes place under conditions for activating the C-S-lyase enzyme of the micro-organism. This may be under anaerobic or aerobic conditions, preferably anaerobic, for 5 to 72 hours and preferably for 12 to 48 hours at pH 6-9, preferably at pH 7-8, with medium to strong stirring and at a temperature of 20 to 50°C and preferably around 30°C. The concentration of the substrate, namely of the complex in the culture medium is from 1 to 100 mmol and preferably 20 to 40 mmol.

In particular, in the case of baker's yeast, it is preferred to operate under anaerobic conditions, for example under an inert gas such as nitrogen, so as to minimize the generation of metabolites of the baker's yeast itself and conversion products of such metabolites, for example by oxidation, and hence to direct fermentation to the production of thiols.

Baker's yeast may be used in the form of a cream or extract. It is preferably fresh, in particular 0 to 18 days old, and advantageously 0 to 8 days old, kept in a refrigerator.

Incubation may be carried out by adding the substrate in one or more steps, preferably progressively, taking into account the kinetics of the enzymatic reaction, so that the latter does not inhibit the activity of the enzyme. With the same idea in mind, it could be possible to consider increasing the quantity of substrate and to extract the volatile thiols as they are produced, so as to increase the yield and rate of the lysis reaction. For this purpose,

volatile organic compounds can be attached to a support, for example a resin, and then eluted. They can also be extracted during incubation in a two-phase medium with the aid of a water-immiscible solvent, preferably of food quality, for example pentane or hexane.

Following incubation, the thiols may be extracted with a solvent, preferably of food quality, for example pentane or hexane, and can be purified by conventional methods known to a person skilled in the art.

As a variant, the thiols formed in the supernatant can be extracted and separated, for example by centrifuging, and then optionally concentrated and dried, for example by spraying or lyophilizing in the presence of a solid support, for example maltodextrin. Drying takes place under moderate conditions, for example at a temperature of $< 70^{\circ}\text{C}$ under vacuum.

The invention also concerns the use of a product of the preceding reaction containing thiols and their metabolites in a food, as it is, in a flavouring composition or as a flavour enhancer.

Such flavours may be incorporated in foods intended for human or animal consumption. In the present specification a micro-organism which can be used in foods is understood to mean any food quality micro-organism, whether this be for human or animal consumption.

The following examples illustrate the invention. In these, percentages and parts are by weight unless indicated to the contrary.

Examples 1 to 4**1.1. Preparation of cream of baker's yeast**

- 5 Commercial cream of baker's yeast, *Saccharomyces cerevisiae*, containing 22-28 % dry matter, was clarified by centrifuging and the supernatant was discarded. The sediment was then mixed with a 0.1M aqueous solution of phosphate buffer at pH 7.5.

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1.2. Preparation of a cysteine-furfural complex or 2-(2-furyl)-1,3-thiazolidine-4-carboxylic acid

- 15 7.7 g (63.6 mmol) of cysteine were dissolved in 200 ml of a 40 % aqueous solution of ethanol in a reactor. A solution of 7 g (72.9 mmol) of furfural in 20 ml of ethanol was then added drop-wise with stirring. The light yellow solution was left stirring and the appearance of a precipitate was
20 observed at the end of 1 hour. After having placed the reactor in an ice bath for 1 hour, the precipitate was separated by filtration, washed with 2 x 50 ml of ethanol and dried under reduced pressure. 10.95 g of a whitish powder of cysteine-furfural complex were thus obtained with
25 a yield of 86.5 %.

- The structure of the compound, verified by electron impact mass spectrography (MS/EI) and by nuclear magnetic resonance of the proton in dimethyl sulphoxide (¹H-NMR)
30 showed the presence of two cis/trans diastereoisomers in a ratio of 1:2.

1.3. Incubation

- 35 100 ml of the preceding cream of whole baker's yeast cells were then placed in a reactor fitted with a pH electrode, a condenser and a magnetic stirrer revolving at 600 rpm. The

reactor was placed in an oil bath thermostatically controlled at 30°C. The reactor was linked to a pH-stat keeping the pH at the chosen value (6.9 or 8) by addition of a 2N aqueous solution of sodium hydroxide. In the case
5 of an anaerobic test, nitrogen was bubbled through the suspension for 15 min by means of a tube in the outlet from the condenser and the reactor was kept under nitrogen for the period of incubation. 0.4 g (2 mmol) and 0.8 g (4 mmol) respectively of 2-(2-furyl)-1,3-thiazolidine-4-
10 carboxylic acid were added to this and 5 ml samples were taken at various incubation times.

1.4. Analysis of bioconversion results

15 After having adjusted the pH of the sample to 4 with a 2N aqueous solution of hydrochloric acid, 500 microlitres of a solution of benzyl thiol in pentane (2000 ppm) were added as an internal standard. After adding 3 g of sodium chloride, the sample was extracted with 3 x 15 ml of
20 diethyl ether, the extracts were separated from the reaction medium by centrifuging (15 min., 5000 rpm), and these were combined, dried over sodium sulphate and concentrated in a Vigreux column to a volume of 2 ml, and the concentrated solution obtained was placed in a freezer
25 at -20°C until analysed.

Analysis by gas chromatography was carried out on a Carlo Erba Mega 2 chromatograph fitted with a cold injector and a flame ionization detector (GC-FID). A flame photometric
30 detector (GC-FPD) was used for the sulphur compounds. The capillary columns were DB-5, DB-1701, DB-Wax and DB-FFAP, 30 m x 0.32 mm, film thickness 0.25 micron, from J & W Scientific, Folsom, USA. The carrier gas was helium (65 kPa) with an addition of nitrogen (40 kPa) for the GC-
35 FID. The retention indices (RI) were calculated by linear interpolation.

The analyses were confirmed by establishing spectra produced by coupling a gas chromatograph with an electron impact mass spectrograph (GC/EI-MS, HP 5890/HP 5971) under the same operating conditions as for GC-FID.

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The results for the principal sulphur compounds relating to aerobic incubation are shown below in table 1 and those concerning anaerobic incubation in table 2.

Table 1

Compound	RI (DB-Wax) , FID	RI (DB-Wax) , FPD	RI (FFAP) , FPD	RI (DB5) , FPD	RI (DB-1701) , FPD
S-methyl thioacetate	1037	1039	1048	-	-
S-ethyl thioacetate	1087	1088	1090	-	-
Thioacetic acid	-	1148	-	-	713
Diethyl disulphide	1208	1192	-	-	-
2-thio-1-ethanol	-	1502	1500	-	-
S-furfuryl-thio thioacetate	2215	2217	-	-	-
FFT	1434	1438	1437	908	1000
Me-FFT	1486	1487	1487	998	1091
Ac-FFT	1768	1769	1770	1159	1276
Di-FFT	2570	2570	2602	1690	1864

Table 2

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Compound	RI (DB-Wax) , FID	RI (DB-Wax) , FPD	RI (FFAP) , FPD	RI (DB5) , FPD	RI (DB-1701) , FPD
FFT	1435	1440	1438	911	1000
Me-FFT	1487	1487	1487	998	1091
Ac-FFT	1768	1768	1769	1159	1276
Di-FFT	-	2570	2600	1689	1864

-: not determined

It was noted that aerobic incubation produced other sulphur compounds in addition to those sought (FFT, Me-FFT, Ac-FFT and Di-FFT). These other compounds probably resulted from the metabolism of cysteine.

Anaerobic incubation mainly produced the compounds sought.

Table 3 below gives the yield of FFT, calculated as a % of the starting complex, as a function of the incubation conditions. In examples 1-4, the yeast used was 4 days old.

Table 3

Example	1	2	3	4
Aerobic	+	-	-	-
Anaerobic	-	+	+	+
pH	6.9	6.9	6.9	8
Concentration of substrate (mmol)	20	20	40	20
Yield of FFT (%) after (days)				
1	25.4	14	2.2	36.9
2	15	27.8	5	27.5
3	11	26	6	16
4	6	24	5	12
7	2	8	6	8

Example 5

Incubation was carried out under the same conditions as for example 2, except for the 8 day yeast. Table 4 below gives the total yield of FFT and its metabolites as well as individual yields.

Table 4

Yield (%)	After 1 day	After 2 days	After 3 days	After 4 days	After 7 days
Total	8.1	16.5	17.4	18.1	16.6
FFT	6.2	15	14	12.5	13.2
Me-FFT	-	0.5	0.5	1	0.5
Ac-FFT	1.9	1	1.9	2.6	0.5
Di-FFT	-	-	1	2	2.4

5 -: not determined

Example 6

10 Incubation was carried out under the same conditions as for example 4, except for the 8 day yeast. Table 5 below gives the total yield of FFT and its metabolites as well as individual yields.

Table 5

Yield (%)	After 1 day	After 2 days	After 3 days	After 4 days	After 7 days
Total	41	32	22	17	24
FFT	37	28	17	13	8
Ac-FFT	2	-	-	-	-
Di-FFT	2	4	5	4	16

15 -: not determined

Example 7

20 Incubation was carried out under the same conditions as for example 2, except that fresh yeast (0 days) and 18 day yeast were used respectively. In order to carry out incubation with an 18 day yeast, the yeast was kept in a refrigerator at 4°C. Just before use, the phosphate buffer

solution was removed by centrifuging and was replaced by the same volume of fresh buffer.

Table 6 below gives the yield of FFT.

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Table 6

FFT Yield (%)	After 1 day	After 2 days	After 3 days	After 4 days	After 7 days
Fresh yeast	1	16	24	-	-
18 day yeast	13	22	22.5	20	9

- : not determined

Examples 8 to 10

10 Proceeding as in example 2, the following complexes were incubated :

8. Cysteine-benzaldehyde and its bioconversion into benzyl thiol was obtained.

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9. 5-methyl-furfuryl-cysteine and its bioconversion into 5-methyl-furfuryl thiol was obtained, and

20 10. 2-methyl-tetrahydrofuranyl-cysteine, which led to 2-methyl-tetrahydrofuran-3-thiol.

Examples 11 to 13

25 Proceeding in a similar manner to that used in example 2, conversion of cysteine-furfural into FFT was obtained by incubation with the following micro-organisms :

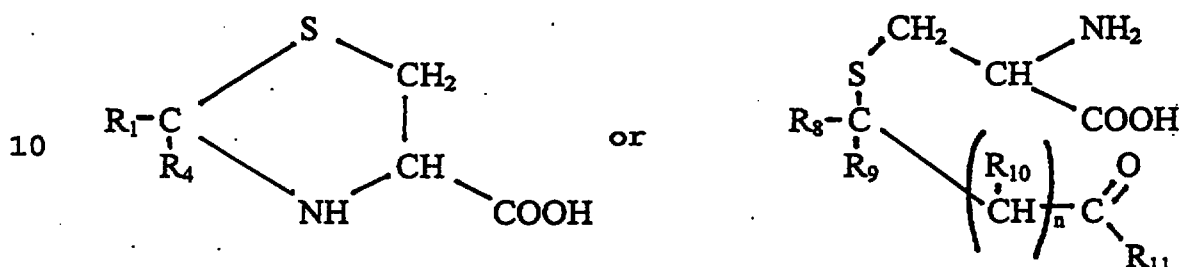
11. *Candida versatilis*

12. *Debaromyces hansenii*

13. *Saccharomyces bayanus*.

Claims

1. Process for the preparation of thiols, in particular natural flavouring thiols, by bioconversion, characterized in that a cysteine S-complex with the formula :



in which :

- R_1 is H, C_{1-7} linear or branched alkyl, phenyl, benzyl, 2- R_2 -2- R_3 -vinyl, 5- R_2 -furan-2-yl, 5- R_2 -thiofuran-2-yl, R_2 and R_3 are similar or different and represent H or CH_3 , R_4 is H, or forms with R_1 a 2- R_5 -5- R_6 -tetrahydrofuran-3-ylidene, or 2- R_5 -5- R_6 -2,3-dihydrofuran-3-ylidene group, in which R_5 and R_6 are similar or different and represent H or CH_3 , or R_4 is a $\text{CH}(\text{OH})\text{R}_7$ group and R_7 represents H, C_{1-4} -alkyl or phenyl, R_8 , R_9 and R_{10} are similar or different and represent H or C_{1-4} -alkyl, R_{11} represents H, C_{1-4} -alkyl, phenyl, C_{1-4} -alkoxy, or C_{1-4} -alkylamino and n is 0 or 1,

is put into contact with a micro-organism which can be used in food, having an enzymatic activity of the beta-C-S-lyase type.

2. Process according to claim 1, characterized in that R_1 is methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, n-hexyl or n-heptyl.



3. Process according to claim 1, characterized in that R_8 to R_{10} is H or methyl and R_{11} is methyl, ethyl, phenyl, methoxy, ethoxy, methylamino or ethylamino.

5 4. Process according to claim 1, characterized in that the preferred compounds liberated by beta-C-S-lyase are selected from furfuryl thiol, benzyl thiol, 5-methylfurfuryl thiol, 2-methylfuran-3-thiol, 2,5-dimethylfuran-3-thiol, 2-methyl-tetrahydrofuran-3-thiol and
10 3-methyl-2-butene-1-thiol.

5. Process according to claim 1, characterized in that the micro-organism used is baker's yeast, *Saccharomyces cerevisiae*.

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6. Process according to claim 1, characterized in that the micro-organism used is selected from brewer's yeast, and the yeasts *Candida versatilis*, *Debaromyces hansenii*, *Saccharomyces bayanus* and *Saccharomyces rouxii*.

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7. Process according to claim 1, characterized in that the micro-organism is a filamentous fungus, in particular *Aspergillus oryzae*, *Penicillium roqueforti* and *Penicillium camemberti*.

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8. Process according to claim 5, characterized in that the incubation reaction with the micro-organism takes place under anaerobic conditions, for 5 to 72 hours and preferably for 12 to 48 hours, and at pH 6-9, preferably at
30 pH 7-8, with medium to strong stirring and at a temperature of 25 to 40°C and preferably around 30°C and that the concentration of the substrate, namely of the complex in the culture medium is from 1 to 100 mmol and preferably 20 to 40 mmol.

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9. Process according to claim 5, characterized in that the baker's yeast is preferably fresh, in particular 0 to 18 days old, and advantageously 0 to 8 days old.

10. Process according to claim 1, characterized in that incubation comprises an addition of the substrate in one or more steps, preferably progressively, taking into account the kinetics of the enzymatic reaction, so that the latter does not inhibit the activity of the enzyme.

11. Process according to claim 1, characterized in that the quantity of substrate is increased and in that the volatile thiols are extracted as they are produced, so as to increase the yield and rate of the enzymatic reaction.

12. Process according to claim 1, characterized in that after incubation, the thiols formed in the supernatant are extracted, separated and purified according to known methods, or they are concentrated and dried in the presence of a solid support under moderate conditions, in particular at a temperature of $< 70^{\circ}\text{C}$ under vacuum.

13. Use of the product obtained by the process according to one of claims 1 to 12, containing thiols and their metabolites as flavouring ingredients.

14. Food intended for animal or human consumption, characterized in that it contains a product obtained by the process according to one of claims 1 to 12 or a flavouring composition containing it.

