

BIOTECHNOLOGICAL PRODUCTION OF OCTANOIC ACID

FIELD OF THE INVENTION

The present invention relates to a biotechnological method of producing octanoic acid. In particular, the method enables an improved yield of octanoic acid produced from ethanol or a mixture of ethanol and acetate using *Clostridium kluyveri*.

BACKGROUND OF THE INVENTION

Octanoic acid also known as caprylic acid is commonly used in several sectors including health promotion, disease control, cosmetics and in industry. For example, it may be used as an antimicrobial pesticide for surface sanitization in the food and dairy industry, and as disinfectant in some healthcare sectors and services. Octanoic acid is also widely used in the commercial production of esters and in perfume and dye manufacturing.

Currently, It is a common industrial chemical, which is produced by oxidation of the C8 aldehyde. Compounds of octanoic acid are found naturally in the milk of various mammals and as a minor constituent of coconut oil and palm kernel oil. However, there is a need for an environmentally friendly and bio-based alternative to produce octanoic acid and salts thereof in a larger scale.

Octanoic acid is well known to be produced as a side product during the chain elongation process using microorganisms which are capable of elongating dilute ethanol and acetate into hexanoic acid. *Clostridium kluyveri* and members of *Firmicute* are particularly useful in the production of hexanoic acid from simple carbon sources such as ethanol. During this process of hexanoic acid, n-caprylate is a minor component side product, if produced at all. The value of n-octanoate is about twice that of hexanoate on a weight basis. Since n-octanoate is considerably more valuable than n-hexanoate by weight, there is a need in the art to produce more octanoate relative to hexanoate from ethanol using the chain elongation method with a microorganism.

However, to be viable and outperform the current methods of producing octanoic acid, not only the yield of octanoic acid produced must be increased but the production must also be cost-effective.

DESCRIPTION OF THE INVENTION

The present invention attempts to solve the problems above by providing a method for the biotechnological means of producing octanoic acid and salts thereof from a carbon source using a microorganism capable of carrying out chain elongation. The carbon source may be ethanol or a mixture of ethanol and acetate. In particular, the microorganism capable of carrying out chain elongation may be *Clostridium kluyveri* or a mixed culture of microorganisms comprising *Clostridium kluyveri*. In particular, the method comprises an essential step of removal of a portion of or all of the octanoic acid and/or salts thereof that is produced from the ethanol or mixture of ethanol and acetate during the fermentation. The step of removal of the produced octanoic acid and/or salts thereof may be carried out during an acclimatisation phase, a production phase, or during both phases. The simultaneous extraction of octanoic acid and salts thereof as the octanoic

acid and salts thereof are produced that plays a significant role in the production of unreported levels of octanoic acid and salts thereof, particularly during fermentation.

During the acclimatisation phase, the cells according to any aspect of the present invention are being accustomed to produce octanoic acid in the fermentation medium in the presence of

5 *Clostridium kluyveri*. During the production phase, the acclimatised cells are capable of producing octanoic acid at a significant yield (i.e. cells are producing octanoic acid at the optimum level).

In one aspect of the present invention, there is provided a method for producing a octanoic acid and/or salts thereof, the method comprising:

- 10 a) contacting a carbon substrate comprising ethanol or a mixture of ethanol and acetate to the aqueous medium comprising *Clostridium kluyveri*;
- b) holding the aqueous medium for a suitable period of time until the *Clostridium kluyveri* produces a significant amount of octanoic acid and/or salts thereof;
- c) separating the octanoic acid and/or salts thereof from the aqueous medium using an extraction medium; and
- 15 d) repeating step (c) continuously as the octanoic acid and/or salts thereof is being formed in a production phase

wherein the extraction medium comprises:

- at least one trialkyl-phosphine-oxide, and at least one alkane.

20 The method according to any aspect of the present invention is particularly advantageous as due to the specific combination of steps (a) to (d), particularly step (d), an unreported amount of octanoic acid is produced.

Octanoic acid and salts thereof refer to and include the undissociated species (n-octanoic acid) and dissociated species (n-octanoate), a carboxylate. Octanoic acid and octanoate are also known as caprylic acid and caprylate respectively. Octanoate can have various counter cations. Examples of
25 suitable cations include, sodium ion, potassium ion, and the like. The method according to any aspect of the present invention produces predominantly octanoic acid and salts thereof. In one example, octanoic acid is produced. In another example, octanoate is produced. In yet another example, octanoic acid and octanoate is produced. There may also be other carboxylates produced in the method according to the present invention, but these are produced in much lower
30 concentrations and are not the target product. The method according to any aspect of the present invention uses *Clostridium kluyveri* at, for example, ambient pressures, reducing capital costs by ensuring a simple bioreactor design.

The term 'carbon substrate' as used herein refers to ethanol alone or a combination of ethanol and acetate with a high ethanol-to-acetate ratio. For example, the high ethanol-to-acetate ratio may be
35 at least at a 5:1 molar ratio.

The term "acetate" as used herein, refers to both acetic acid and salts thereof, which results inevitably, because as known in the art, since the microorganisms work in an aqueous environment, and there is always a balance between salt and acid present.

5 The term "contacting", as used herein, means bringing about direct contact between the cell according to any aspect of the present invention and the medium comprising the carbon substrate in step (a). For example, the cell, and the medium comprising the carbon substrate may be in different compartments in step (a). they may then be brought together in a fermenter.

10 The term "an aqueous solution" or "medium" comprises any solution comprising water, mainly water as solvent that may be used to keep the cell according to any aspect of the present invention, at least temporarily, in a metabolically active and/or viable state and comprises, if such is necessary, any additional substrates. The person skilled in the art is familiar with the preparation of numerous aqueous solutions, usually referred to as media that may be used to keep inventive cells, for example LB medium in the case of *E. coli*, ATCC1754-Medium may be used in the case of *C.ljungdahlii* and EvoDM24 medium (pH 5.5; 0.429 g/L Mg-acetate, 0.164 g/l Na-acetate, 0.016 g/L Ca-acetate, 2.454 g/l K-acetate, 0.107 mL/L H₃PO₄ (8.5%), 0.7 g/L NH₄acetate, 0.35 mg/L Co-acetate, 1.245 mg/L Ni-acetate, 20 µg/L d-biotin, 20 µg/L folic acid, 10 µg/L pyridoxine-HCl, 50 µg/L thiamine-HCl, 50 µg/L Riboflavin, 50 µg/L nicotinic acid, 50 µg/L Ca-pantothenate, 50 µg/L Vitamin B12, 50 µg/L p-aminobenzoate, 50 µg/L lipoic acid, 0.702 mg/L (NH₄)₂Fe(SO₄)₂ x 4 H₂O, 1 mL/L L-cysteine (93,5 mM), 20 mL/L ethanol, 0.37 g/L acetic acid) used in the case of *Clostridium kluyveri*.
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20 It is advantageous to use as an aqueous solution a minimal medium, i.e. a medium of reasonably simple composition that comprises only the minimal set of salts and nutrients indispensable for keeping the cell in a metabolically active and/or viable state, by contrast to complex mediums, to avoid dispensable contamination of the products with unwanted side products. For example, M9 medium may be used as a minimal medium.

25 The *Clostridium kluyveri* (DSM555) cells are incubated with the carbon substrate sufficiently long enough (i.e. suitable period of time), to produce the desired product, octanoic acid and salts thereof. For example for at least 1, 2, 4, 5, 10 or 20 hours.

30 In one example, the *C.kluyveri* cells according to any aspect of the present invention, particularly step (b) are incubated with the carbon substrate for at least two phases, an acclimation phase, and a production phase for producing octanoic acids and/or salts thereof. Octanoic acids and/or salts thereof are produced during both phases. For example, the carbon substrate according to any aspect of the present invention is brought into contact with *C.kluyveri* cells and during the acclimation phase (e.g., for at least 1 day), a desired amount of octanoic acid and/or salts thereof are produced (i.e. the optimal rate of production of octanoic acid and/or salts thereof) where the
35 *C.kluyveri* cells reach its optimal, efficient level of chain elongation. During the production phase, the cells according to any aspect of the present invention continue to produce octanoic acid and/or salts thereof at the optimal level.

In step (b) according to any aspect of the present invention, the cells are held and maintained at a pH of 5-8, particularly 5-7.5, 5-7, 5-6.5, 5-6, 5-5.5, and at a temperature of 10 to 42 °C, preferably

30 to 40 °C, in particular, 32 to 38 °C. The temperature and pH chosen must be such that the cells according to any aspect of the present invention remain catalytically competent and/or metabolically active. During this step, octanoic acid and/or salts thereof are produced to a desired yield and constantly produced at the desired yield.

- 5 The produced octanoic acid and/or salts thereof is in part or completely continuously removed or extracted from the aqueous medium, also known as the reaction mixture where octanoic acid and/or salts thereof are produced through fermentation in the presence of the *Clostridium kluyveri* and the carbon substrate, ethanol or a mixture of ethanol and acetate. This step allows for the increased yield of octanoic acid and/or salts thereof according to any aspect of the present
10 invention. In particular, the octanoic acid and/or salts thereof are extracted simultaneously as the octanoic acid and/or salts are produced.

In one example, the extracting medium is added to the aqueous medium comprising the cells according to any aspect of the present invention and the carbon substrate.

- In particular, in step (c), the octanoic acid and/or salts thereof is separated from the aqueous
15 medium using an extraction medium. More in particular, the extraction method according to any aspect of the present invention allows for an increase in yield relative to the amount of extractants used. For example, less than 50% by weight of extracting medium may be used to extract the same amount of octanoic acids and/or salts thereof compared to when only pure alkanes were used. Therefore, with a small volume of extracting medium, a larger yield of octanoic acids and/or
20 salts thereof may be extracted. The extracting medium is also not harmful to microorganisms. Accordingly, the extracting medium according to any aspect of the present invention may be present when the octanoic acids and/or salts thereof is biotechnologically produced. Further, the octanoic acids and/or salts thereof can then be easily separated from the extraction medium using any method known in the art such as distillation, funnel, column, and the like. After the separation
25 of the octanoic acid and/or salts thereof from the extraction medium, the extracting medium may be easily recycled.

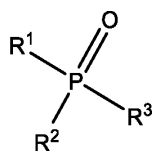
- The extracting medium may also be referred to as the 'extraction medium'. The extraction medium may be used for extracting/ isolating the octanoic acid and/or salts thereof produced according to any aspect of the present invention from the aqueous medium wherein the octanoic acid and/or salts thereof was
30 originally produced. At the end of the extracted step, excess water from the aqueous medium may be removed thus resulting in the extracting medium containing the extracted octanoic acid and/or salts thereof. The extracting medium may comprise a combination of compounds that may result in an efficient means of extracting the octanoic acid and/or salts thereof from the aqueous medium. In particular, the extraction medium may comprise: (i) at least alkane comprising at least 12 carbon atoms, and (ii) at least
35 one molecule trialkyl-phosphine oxide (TAPO).

- The extracting medium according to any aspect of the present invention may efficiently extract the octanoic acid and/or salts thereof into the alkane- alkyl-phosphine oxide extracting medium. This extracting medium of a mixture of alkyl-phosphine oxides and at least one alkane may be considered
40 suitable in the method according to any aspect of the present invention as the mixture works efficiently in

extracting octanoic acid and/or salts thereof in the presence of a fermentation medium. In particular, the mixture of alkyl-phosphine oxides and at least one alkane may be considered to work better than any method currently known in the art for extraction of octanoic acid as it does not require any special equipment to be carried out and it is relatively easy to perform with a high product yield.

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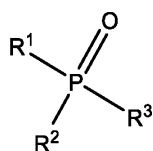
In particular, the alkyl-phosphine oxide also known as a tri-alkyl-phosphine oxide (TAPO) used in the method according to any aspect of the present invention is selected from an alkyl-phosphine oxide of general formula 1



general formula 1

- 10 with R¹, R² and R³ selected from alkyl radicals, preferably linear alkyl radicals, containing 4 to 18, preferably 6 to 12, carbon atoms,
with the proviso, that at least two of R¹, R² and R³ differ from each other.

- More in particular, the alkyl-phosphine oxide is selected from an alkyl-phosphine oxide of general formula
15 1 with R¹, R² and R³ selected from alkyl radicals, preferably linear alkyl radicals, containing 8 to 10, particularly 8 or 10, carbon atoms, more particularly with the proviso, that referring to all alkyl-phosphine oxide of general formula 1 contained in the extracting medium, the molar ratio of all alkyl radicals containing 8 and 10 carbon atoms is in the range of from 1.0:2.0 to 2.0:1.0, particularly from 1.0:1.5 to 1.5:1.0, even more particularly from 1.0:1.2 to 1.2:1.0. Even more in particular, the alkyl-phosphine oxide
20 in the extracting medium in the method according to any aspect of the present invention is an alkyl-phosphine oxide of general formula 1



general formula 1

- with R¹, R² and R³ selected from linear alkyl radicals with 6 to 12, carbon atoms and wherein one alkyl radical has 8 carbon atoms and the other 10 carbon atoms,
25 with the proviso, that at least two of R¹, R² and R³ differ from each other.

- Even more in particular, in the extracting medium in the method according to any aspect of the present invention the alkyl-phosphine oxide accounts for at least 50 wt.-%, particularly at least 80 wt.-%, even more particularly at least 90 wt.-%, the most particularly at least 97 wt.-%, of the total extracting medium.
30

- In one example, the extracting medium according to any aspect of the present invention may further comprise other than the alkyl-phosphine oxide, a second organic component. The second organic component contains at least 12 carbons. In particular, the alkane may comprise at 12- 18 carbon atoms. The second organic component is an alkane linear or branched that may be selected from the
35 group consisting, tetradecane, pentadecane, hexadecane, heptadecane, octadecane and squalene or

mixtures of alkanes such as white mineral oil (Fragoltherm-Q-32-N). Furthermore, the second organic component may comprise of an aromatic hydrocarbon that may be selected from the group consisting, diisopropylbiphenyl, partly hydrogenated terphenyl, dibenzyltoluol and diisopropylnaphthalene or a mix of aromatic solvents such as Solvesso 200. Another possibility is to use an alcohol that may be selected
5 from the group consisting, oleyl alcohol, 2-octyldodecanol and 2-hexyldodecanol as the second organic component.

The weight ratio of the alkyl-phosphine oxide to alkane in the extracting medium according to any aspect of the present invention preferably is between 1:100 to 100:1. Even more in particular, the weight ratio of
10 the alkyl-phosphine oxide to alkane may be selected within the range of from 1:2 to 50:1, more preferably from 1:1 to 97:3. In the example, the alkane may be hexadecane and therefore the weight ratio of the alkyl-phosphine oxide to hexadecane may be about 97:3.

The extracting medium according to any aspect of the present invention may efficiently extract the
15 octanoic acid into the extracting medium. This extracting medium of a mixture of at least one alkyl-phosphine oxide containing at least two different alkyl radicals per alkyl-phosphine oxide molecule, and at least one alkane may be considered suitable in the method according to any aspect of the present invention as the mixture works efficiently in extracting the desired octanoic acid. The alkane may be a straight or a branched alkane. In one example, the alkane may be a branched alkane and the branched
20 alkane may be squalene.

In another example, the extracting medium of a mixture of at least one alkyl-phosphine oxide containing at least two different alkyl radicals per alkyl-phosphine oxide molecule and at least one partially hydrogenated aromatic hydrocarbon may be considered suitable in the method according to any aspect
25 of the present invention as the mixture works efficiently in extracting octanoic acids and salts thereof. In particular, the mixture of at least one alkyl-phosphine oxide containing at least two different alkyl radicals per alkyl-phosphine oxide molecule and at least one partially hydrogenated aromatic hydrocarbon. Further, the extracting medium according to any aspect of the present invention in combination with alkane or partially hydrogenated aromatic solvent is also not toxic for microorganisms.

30 The term 'about' as used herein refers to a variation within 20 percent. In particular, the term "about" as used herein refers to +/- 20%, more in particular, +/-10%, even more in particular, +/- 5% of a given measurement or value.

35 The source of acetate and/or ethanol may vary depending on availability. In one example, the ethanol and/or acetate may be the product of fermentation of synthesis gas or any carbohydrate known in the art. In particular, the carbon source for acetate and/or ethanol production may be selected from the group consisting of alcohols, aldehydes, glucose, sucrose, fructose, dextrose, lactose, xylose, pentose, polyol, hexose, ethanol and synthesis gas.

40 Mixtures of sources can be used as a carbon source.

Even more in particular, the carbon source may be synthesis gas. The synthesis gas may be converted to ethanol and/or acetate in the presence of at least one acetogenic bacteria.

- Additional substrate of ethanol or ethanol and acetate may be added throughout the fermentation process to keep the production of octanoic acid and/or salts thereof going. In one example, additional substrate is periodically added. In another example, additional substrate is continuously added, or a combination thereof during the fermentation or production of octanoic acid and/or salts thereof. It is desirable not to overfeed (feed more than the rate of chain elongation, which is equal to the extraction rate) the cells according to any aspect of the present invention.
- 5 In one example, the significant amount of octanoic acid and/or salts thereof in step (c) refers to about 100ppm. In particular, at least about 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275 or 300ppm. Octane products can be detected by methods known in the art. For example, octane products are detected by gravimetric methods, spectroscopic methods (e.g., nuclear magnetic resonance (NMR) spectroscopic methods), or mass spectrometry methods (e.g., gas or liquid chromatography/mass spectrometry methods).
- 10 15

In one example, other than octanoic acid and salts thereof, hexanoic acid and/or salts thereof are also produced and the aqueous medium further comprises other microorganisms like *Clostridium autoethanogenum*.

- According to another aspect of the present invention, there is provided a method of improving yield of octanoic acid and/ or salts thereof produced in a product composition, the method comprising the step of:
- 20

i) simultaneously extracting the octanoic acid and/or salts thereof as it is being formed in the product composition,

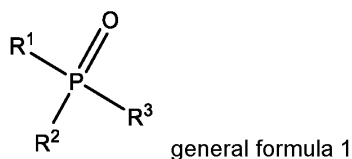
- wherein the octanoic acid and or salts thereof is formed in the presence of *Clostridium kluyveri* and a substrate comprising ethanol or a mixture of ethanol and acetate.
- 25

In particular, the yield of octanoic acid and/or salts thereof in the product composition is improved by at least a factor of about 1.5, particularly 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9 or 3.

In particular, the extraction is carried out using an extraction medium comprising:

- 30 - at least one trialkyl-phosphine-oxide, and at least one alkane.

In one example, the alkane comprises 12 to 18 carbon atoms. In another example, the extraction medium comprises at least one trialkyl-phosphine-oxide, and at least one alkane. In particular, the trialkyl-phosphine oxide is selected from a trialkyl-phosphine oxide of general formula 1



with R^1 , R^2 and R^3 selected from alkyl radicals containing 4 to 18, preferably 6 to 12, carbon atoms, with the proviso, that at least two of R^1 , R^2 and R^3 differ from each other.

EXAMPLES

- 5 The foregoing describes preferred embodiments, which, as will be understood by those skilled in the art, may be subject to variations or modifications in design, construction or operation without departing from the scope of the claims. These variations, for instance, are intended to be covered by the scope of the claims.

Example 1

- 10 *Cultivation of Clostridium autoethanogenum and Clostridium kluyveri in a one-phase system to produce octanoate*

The bacterium *Clostridium kluyveri* (DSM555) was cultivated in a co culture with *Clostridium autoethanogenum* (DSM10061) for the biotransformation of ethanol and acetate to medium chain fatty acids. All cultivation steps were carried out under anaerobic conditions, the preculture in a stainless steel
15 bubble column loop reactor and the main culture in a pressure-resistant glass bottle that can be closed airtight with a butyl rubber stopper.

- For the pre-cultivation, the homoacetogenic bacterium, *Clostridium autoethanogenum* was cultivated together with the chain elongating bacterium *Clostridium kluyveri* as a coculture on synthesis gas in a mineral medium. The cultivation was carried out under anaerobic conditions in a pressure-resistant
20 stainless steel bubble column loop reactor.

- The cultivation was run at 37°C and an overpressure of 2 bar as a continuous fermentation with continuous feeding of 300 L/h of a mixture of water, substrates, salts, trace elements and vitamins. The pH was automatically hold at 5.80 with ammonia feeding. The outlet stream of 300 L/h out of the fermenter was for 98,2% as permeate with cell retention and for 1,8% as purge without cell retention. The
25 gas was discharged into the medium through a sparger with a ventilation rate of ~ 1000 L/h as a gas mixture of 62,5% H_2 and 37,5% CO_2 .

- The media feed consisted of 0.004 g/L Mg-acetate x 4 H_2O , 0.164 g/L Na-acetate, 0.016 g/L Ca-acetate, 0.245 g/L K-acetate, 0.107 mL/L H_3PO_4 (8.5%), 0.35 mg/L Co-acetate, 1.245 mg/L Ni-acetate x 4 H_2O , 20 μ g/L d-biotin, 20 μ g/L folic acid, 10 μ g/L pyridoxine-HCl, 50 μ g/L thiamine-HCl, 50 μ g/L Riboflavin, 50 μ g/L
30 nicotinic acid, 50 μ g/L Ca-pantothenate, 50 μ g/L Vitamin B12, 50 μ g/L p-aminobenzoate, 50 μ g/L lipoic acid, 2.109 mg/L $(NH_4)_2Fe(SO_4)_2$ x 6 H_2O , 10.69 mg/L potassium thioacetate, 6,73 g/L ethanol and NH_3 for pH regulation.

The culture was previously inoculated with cells from fresh cultures of *C. autoethanogenum* and *C.kluyveri* and was already running since > 30.000 h full continuously as stable coculture at an optical

density (OD_{600nm}) of ~ 14 . Fresh medium was continuously feeded into the reactor and fermentation broth continuously removed from the reactor with a dilution rate of $2.8 d^{-1}$.

For the main culture 180 ml of the same anaerobic mineral medium as used in the preculture with a higher ethanol concentration (20 mL/L ethanol) and additional 5 g/L NH_4 acetate and 0.15 g/L NH_4HCO_3 were filled in a 500 mL bottle and inoculated with as many cell broth from the preculture to reach an OD_{600nm} of 0.15. The bottle with the one-layer aqueous phase was then cultivated at $37^\circ C$, 150 rpm and with a gas atmosphere of 10 % H_2 and 90 % N_2 in an open water bath shaker for 67 h. The gas atmosphere in the bottle was flushed once a day for 10 min with pure N_2 .

During cultivation several 5 mL samples from the aqueous phase were taken to determinate OD_{600nm} , pH and product formation. The determination of the product concentrations in the aqueous phase was performed by semiquantitative 1H -NMR spectroscopy and high performance liquid chromatography (HPLC).

During the cultivation of 67 h in the aqueous phase the concentration of acetate decreased from 4.3 g/L to 1.4 g/L and the concentration of ethanol decreased from 14.0 g/L to 9.1 g/L. During the same time 2.1 g/L butyrate, 3.6 g/L hexanoate and 0.04 g/L octanoate were built.

Example 2

Cultivation of Clostridium autoethanogenum and Clostridium kluyveri in a two-phase system to produce octanoate

The bacterium *Clostridium kluyveri* (DSM555) was cultivated in a co culture with *Clostridium autoethanogenum* (DSM10061) for the biotransformation of ethanol and acetate to medium chain fatty acids. For the *inSitu* extraction of the produced acids the aqueous phase was overlayed with a mixture of FragoItherm Q-32-N with trialkylphosphineoxide (TAPO). All cultivation steps were carried out under anaerobic conditions, the preculture in a stainless steel bubble column loop reactor and the main culture in a pressure-resistant glass bottle that can be closed airtight with a butyl rubber stopper.

For the pre-cultivation the homoacetogenic bacterium *Clostridium autoethanogenum* was cultivated together with the chain elongating bacterium *Clostridium kluyveri* as a coculture on synthesis gas in a mineral medium. The cultivation was carried out under anaerobic conditions in a pressure-resistant stainless steel bubble column loop reactor.

The cultivation was run at $37^\circ C$ and an overpressure of 2 bar as a continuous fermentation with continuous feeding of 300 L/h of a mixture of water, substrates, salts, trace elements and vitamins. The pH was automatically hold at 5.80 with ammonia feeding. The outlet stream of 300 L/h out of the fermenter was for 98,2% as permeate with cell retention and for 1,8% as purge without cell retention. The gas was discharged into the medium through a sparger with a ventilation rate of $\sim 1000 L/h$ as a gas mixture of 62,5% H_2 and 37,5% CO_2 .

The media feed consisted of 0.004 g/L Mg-acetate x 4 H_2O , 0.164 g/L Na-acetate, 0.016 g/L Ca-acetate, 0.245 g/L K-acetate, 0.107 mL/L H_3PO_4 (8.5%), 0.35 mg/L Co-acetate, 1.245 mg/L Ni-acetate x 4 H_2O , 20 $\mu g/L$ d-biotin, 20 $\mu g/L$ folic acid, 10 $\mu g/L$ pyridoxine-HCl, 50 $\mu g/L$ thiamine-HCl, 50 $\mu g/L$ Riboflavin, 50 $\mu g/L$ nicotinic acid, 50 $\mu g/L$ Ca-pantothenate, 50 $\mu g/L$ Vitamin B12, 50 $\mu g/L$ p-aminobenzoate, 50 $\mu g/L$ lipoic

acid, 2.109 mg/L $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \times 6 \text{ H}_2\text{O}$, 10.69 mg/L potassium thioacetate, 6,73 g/L ethanol and NH_3 for pH regulation.

The culture was previously inoculated with cells from fresh cultures of *C. autoethanogenum* and *C. kluyveri* and was already running since > 30.000 h full continuously as stable coculture at an optical
5 density ($\text{OD}_{600\text{nm}}$) of ~ 14. Fresh medium was continuously feeded into the reactor and fermentation broth continuously removed from the reactor with a dilution rate of 2.8 d^{-1} .

For the main culture 180 ml of the same anaerobic mineral medium as used in the preculture with a higher ethanol concentration (20 mL/L ethanol) and additional 5 g/L $\text{NH}_4\text{acetate}$ and 0.15 g/L NH_4HCO_3 were filled in a 500 mL bottle and inoculated with as many cell broth from the preculture to reach an
10 $\text{OD}_{600\text{nm}}$ of 0.15. The aqueous phase was then overlayed with 20 ml anaerobisized extracting agent (20% trialkylphosphineoxide (TAPO) in Fragoltherm Q-32-N). The bottle with the two-layer phases was then cultivated at 37°C , 150 rpm and with a gas atmosphere of 10 % H_2 and 90 % N_2 in an open water bath shaker for 67 h. The gas atmosphere in the bottle was flushed once a day for 10 min with pure N_2 .

During cultivation several 5 mL samples from both, the aqueous and the organic phase, were taken to
15 determinate $\text{OD}_{600\text{nm}}$, pH und product formation. The determination of the product concentrations in the aqueous phase was performed by semiquantitative $^1\text{H-NMR}$ spectroscopy and high performance liquid chromatography (HPLC) and in the organic phase by gas chromatography (GC).

During the cultivation of 67 h in the aqueous phase the concentration of acetate decreased from 4.2 g/L to 0.3 g/L and the concentration of ethanol decreased from 13.1 g/L to 5.0 g/L. During the same time 1.1
20 g/L butyrate, 5.7 g/L hexanoate and 0.01 g/L octanoate were built. In the organic phase the concentrations raised from 0 g/L to 0.75 g/L for ethanol, 0 g/L to 0.50 g/L for butyrate, 0 g/L to 25.7 g/L for hexanoate and 0 g/L to 1.08 g/L for octanoate. Acetic acid was not found.

CLAIMS

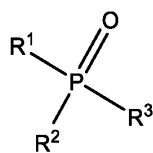
1. A method for producing octanoic acid and/or salts thereof, the method comprising:
- a) contacting a carbon substrate comprising ethanol or a mixture of ethanol and acetate to the aqueous medium comprising *Clostridium kluyveri*;
- 5 b) holding the aqueous medium for a suitable period of time until the *Clostridium kluyveri* produces a significant amount of octanoic acid and/or salts thereof;
- c) separating the octanoic acid and/or salts thereof from the aqueous medium using an extraction medium; and
- d) repeating step (c) continuously as the octanoic acid and/or salts thereof is being formed,
- 10 wherein the extraction medium comprises:
- at least one trialkyl-phosphine-oxide, and at least one alkane.
2. The method according to claim 1, wherein the alkane comprises 12 to 18 carbon atoms.
- 15 3. The method according to either claim 1 or 2, wherein the trialkyl-phosphine oxide is selected from a trialkyl-phosphine oxide of general formula 1
- general formula 1
- with R¹, R² and R³ selected from alkyl radicals containing 4 to 18, preferably 6 to 12, carbon atoms,
- 20 with the proviso, that at least two of R¹, R² and R³ differ from each other.
4. The method according to claim 3, wherein the trialkyl-phosphine oxide is selected from a trialkyl-phosphine oxide of general formula 1 with R¹, R² and R³ selected from alkyl radicals containing 8 to 10 carbon atoms.
- 25 5. The method according to claim 4, wherein the molar ratio of all alkyl radicals containing 8 and 10 carbon atoms in the trialkyl-phosphine oxide is in the range of from 1.0:2.0 to 2.0:1.0.
6. The method according to any one of the preceding claims, wherein ethanol alone or ethanol and acetate are further added as substrates.
- 30 7. The method according to any one of the preceding claims, wherein the significant amount of octanoic acid in step (c) refers to about 100ppm.
- 35 8. The method according to any one of the preceding claims, wherein other than octanoic acid and salts thereof, hexanoic acid is also produced and the aqueous medium further comprises other microorganisms like *Clostridium autoethanogenum*.

9. A method of improving yield of octanoic acid and or salts thereof produced in a product composition, the method comprising the step of:
- i) simultaneously extracting the octanoic acid and/or salts thereof as it is being formed in the product composition,
- 5 wherein the octanoic acid and or salts thereof is formed in the presence of *Clostridium kluyveri* and a substrate comprising ethanol or a mixture of ethanol and acetate and the extraction is carried out using an extraction medium comprising:
- at least one trialkyl-phosphine-oxide, and at least one alkane.

10. The method according to claim 9, wherein the yield of octanoic acid and/or salts thereof in the product composition is improved by at least a factor of about 1.5

11. The method according to either claim 9 or 10, wherein the alkane comprises 12 to 18 carbon atoms.

12. The method according to any one of the claims 9 to 11, wherein the trialkyl-phosphine oxide is selected from a trialkyl-phosphine oxide of general formula 1



general formula 1

- with R¹, R² and R³ selected from alkyl radicals containing 4 to 18, preferably 6 to 12, carbon atoms,
- 20 with the proviso, that at least two of R¹, R² and R³ differ from each other.

13. The method according to claim 12, wherein the trialkyl-phosphine oxide is selected from a trialkyl-phosphine oxide of general formula 1 with R¹, R² and R³ selected from alkyl radicals containing 8 to 10 carbon atoms.
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