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## (54) Title: CO-CULTIVATION OF PROPIONIBACTERIUM AND YEAST

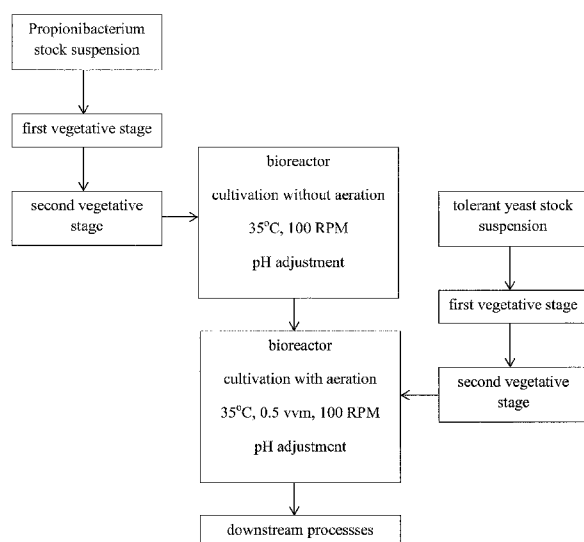


FIG. 3

(57) Abstract: The invention provides a fungal cell, such as a yeast cell, capable of growing in co-cultivation with Propionibacterium. Also provided are methods of producing such cells and fermentation processes using the fungal cell of the invention and Propionibacterium in co-cultivation. Such co-cultivation significantly reduces the chemical oxygen demand load of the waste fermentation broth.

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## CO-CULTIVATION OF PROPIONIBACTERIUM AND YEAST

### 5 Field of the invention

The present invention is in the field of bioprocess technology, in particular, of bioprocesses using co-cultures of yeast and propionibacterium (*i.e.*, *Propionibacterium sp.*).

### Background of the invention

10 Waste streams with a high organic chemical oxygen demand (COD) are an ecological burden that has to be dealt with accordingly. These waste streams can be by-products of different industries such as dairy, fruit and vegetable or sugar processing industries. An example is whey that is produced by the dairy industry as a by-product resulting from cheese making. This process results in high volumes of whey (sweet or sour) that have a high COD but on the other hand cannot be easily/economically utilised otherwise, due to  
15 the low relatively low content of useful dry content, e.g. milk protein and lactose.

Depending on the process of cheese manufacture the COD value of leftover whey can range from 35,000 mg O<sub>2</sub>/L to 100,000 mg O<sub>2</sub>/L. The main contributor of the high organic load is lactose, which can represent up to 90% of the COD value. In the case of sour whey the presence of lactic acid even increases this problem. Lactose also represents about 75%  
20 of the whey dry matter and as such can be exploited by processes utilizing microorganisms. Several solutions of whey utilisation have been studied, such as bioethanol and biogas production, extraction of lactose or proteins, production of organic acids or biomass, but there is still a need of an economical way of whey utilisation.

WO 2011/140649 describes the utilization of whey by a mixed culture of lactic acid  
25 bacteria and yeast for the production of edible biomass. The process can successfully decrease the COD load of whey, but the final product has little commercial value.

Whey can be used for the cultivation of bacteria from the genus *Propionibacterium sp.*, belonging to the order of Actinomycetales (Bergey's Manual of Systematic Bacteriology (1st Edition, 1986)). *Propionibacterium sp.* are known producers of valuable products such  
30 as organic acids (propionic and acetic acid), vitamin B12 and bifidogenic compounds. While *Propionibacterium sp.* can be successfully cultivated on whey they cannot lower the COD load satisfactorily as they produce organic acids which accumulate in the fermented (spent) whey and contribute significantly toward the final COD load. In the following, the terms "*Propionibacterium sp.*" and "propionibacterium" are used interchangeably.

35 Co-cultures of *Propionibacterium sp.* and other bacteria are known to be able to decrease the COD load of the spent medium (Miyano et al., 2000), but such processes have the disadvantage of not being food grade and as such being exempt from being used inside food processing plants.

The metabolites produced by *Propionibacterium sp.* are known to inhibit the growth of other microorganisms, especially fungi, and their use in food-spoilage prevention is well known. US 5,260,061 describes the application of *Propionibacterium sp.* metabolites for food applications to inhibit the growth of yeast. WO 2008/030089 describes co-cultivation of *Propionibacterium sp.* with yeast for the purpose of obtaining good flavour/aroma characteristics in the cheese making process. In this case, *Propionibacterium sp.* was used to control and finally inhibit the growth of yeast in the mixed culture as the yeast cell applied was not tolerant to growth-inhibiting substances from *Propionibacterium sp.*.

In view of the above stated prior art, it is an object of the invention to provide novel bioprocesses for producing valuable biotechnological products from sweet or sour whey where the final spent fermentation media exhibit a relatively low COD. Another object of the invention is to provide fungal cells useful in processes of the invention.

### Summary of the invention.

The present invention meets the objectives stated above by providing novel fungal cells, preferably yeast cells, which are capable of growing in co-cultivation with propionibacterium. Such fungal cells or cells may be characterized/defined by their ability to grow on stationary-phase supernatant (spent medium) resulted after propionibacterium cultivation. The present invention also relates to biotechnological processes using such fungal cells which can grow in the presence of said medium in co-culture with propionibacterium.

Hence, a first aspect of the present invention relates to fungal cell capable of growing in a stationary-phase supernatant of a propionibacterium cultivation.

Such a fungal cell can reproducibly be obtained by mutagenesis/selection procedures described in this patent application. Fungal cells, in particular yeast cells, having the capability of growing in a stationary-phase supernatant of a *propionibacterium* cultivation are useful for the reduction of the COD load of waste materials from fermentation processes according to the invention. Such fungal cells (in particular such yeast cells) were heretofore not available to the person skilled in the art.

Fungal cells of the invention can easily be identified and distinguished from other fungal cells by procedures disclosed herein below. In particular, a well-defined cultivation medium and well defined cultivation conditions of propionibacterium therein are disclosed for testing the capability of a fungal cell of "growing in a stationary-phase supernatant of a propionibacterium cultivation", according to the invention.

Hence, in a preferred embodiment, the stationary-phase supernatant, (to be used for testing whether the fungal cell is one according to the invention) is obtained from a stationary culture of *Propionibacterium freudenreichii* cultivated under anaerobic conditions at 35°C and pH 6.5 in a medium consisting of 60 g/L sweet whey powder, 5 g/L yeast extract, and 40 mg/L calcium D-pantothenate. Preferably, the sweet whey powder has food-grade quality, and preferably it contains at least 63%wt. lactose, at least 10%wt. protein and at most 5%wt. water.

The capability of growing in a stationary-phase supernatant of a propionibacterium cultivation, the cultivation of fungal (yeast) culture can be defined in terms of the maximum growth rate achieved by the fungal cell when growing in a stationary-phase supernatant of a propionibacterium cultivation. Hence, in another preferred embodiment, said capability of growing of said fungal cell in a stationary-phase supernatant of a propionibacterium cultivation is defined as the capability of said fungal cell of growing on said stationary-phase supernatant at a maximum growth rate ( $\mu_{\max}$ ) of at least  $0.02 \text{ h}^{-1}$ . Methods of determining the growth rate of a microorganism in culture are well known in the art.

The stationary culture state of a propionibacterium culture may be indicated by a constant concentration of acetic acid and propionic acid in the culture medium over time when the culture is not exposed to aeration. In another embodiment, the stationary state of the propionibacterium culture is indicated by a constant cell density of propionibacterium (in  $\text{g(DW)/L}$ ) over time.

In a preferred embodiment, the ability of the fungal cell to grow in a stationary-phase supernatant of a propionibacterium cultivation is tested in the test method described in Example 2, herein below.

The preferred fungal cell is a yeast cell.

In one embodiment, the yeast cell is the yeast cell deposited on 14.1.2014 at Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) under accession number DSM 28271.

A second aspect of the invention relates to a process for producing a biotechnological product, said process comprising co-cultivation of propionibacterium and a fungal cell in a cultivation medium. The fungal cell is preferably capable of growing in a stationary-phase supernatant of a propionibacterium cultivation.

The fungal cell is preferably a yeast cell, such as a fungal cell according to the invention, as described hereinabove and below.

The biotechnological product is preferably one which is produced by propionibacterium.

A preferred biotechnological product is vitamin B12.

In a preferred embodiment, the process includes a first phase with no aeration followed by a second phase in which aeration occurs. Preferably, both the phases are at least 24 hours, or 48 hours, or 72 hours long.

In a preferred embodiment, at least 90% of the growth of the propionibacterium [in % gram dry weight] occurs during said first phase without aeration.

In another preferred embodiment, at least 90% of the growth of the fungal cell [in % gram dry weight] occurs during said second phase in which aeration occurs.

In another preferred embodiment, the chemical oxygen demand (COD) of the cultivation medium is reduced to a level of equal to or less than 25%, preferably equal to or less than 10%, or equal to or less than 1%, of the initial COD of the cultivation medium.

5 In another preferred embodiment, the cultivation medium is (or comprises) whey. The cultivation medium is or comprises, e.g., sweet whey, or sour whey.

Preferably, in processes of the invention, said fungal cell is added at or after the point in time when said propionibacterium has reached at least 90% of its maximum cell density [g(DW)/L] in the culture. In other embodiments of the process of the invention, the fungal cell is added when or after the propionibacterium has reached its stationary growth phase.

10 The invention further relates to a method of making a propionibacterium-tolerant fungal strain, such as a propionibacterium-tolerant yeast strain. The method comprises:

1. Obtaining a starting fungal strain.
  2. Exposing the starting fungal strain to a mutagenic agent and/or to a mutagenic condition.
  - 15 3. Cultivating the exposed fungal strain of step 2 in the presence of first concentrations of acetic acid and/or (preferably "and") propionic acid.
  4. Optionally exposing said cultivated fungal strain of step 3 to a mutagenic agent and/or to a mutagenic condition and cultivating said optionally exposed fungal strain in the presence second concentrations of acetic acid and/or (preferably "and")  
20 propionic acid, wherein said second concentrations are preferably higher than said first concentrations, respectively.
  5. Exposing the cultivated fungal strain of step 3 or 4 to a mutagenic agent and/or to a mutagenic condition.
  6. Cultivating the exposed fungal strain of step 6 in a medium comprising stationary-phase supernatant of a propionibacterium cultivation.  
25
  7. Optionally repeating step 7 with increasing concentrations of the stationary-phase supernatant of a propionibacterium cultivation.
  8. Thereby obtaining a fungal cell capable of growing in a stationary-phase supernatant of a propionibacterium cultivation.
- 30 A preferred mutagenic agent in accordance with the invention is ethyl methanesulfonate. Any other mutagenic agent, i.e. chemical or physical can however be used which increases the frequency of mutation occurrence. Known mutagenic agents which can be used in the above method include: Reactive oxygen species (ROS), such as superoxide, hydroxyl radicals and hydrogen peroxide; deaminating agents, for example nitrous acid; polycyclic aromatic hydrocarbon (PAH); alkylating agents such as ethylnitrosourea or methyl  
35 methanesulfonate; guanine; nitrosamines; nitrosoguanidine; mustard gas; vinyl chloride; aromatic amines; amides; 2-acetylaminofluorene; alkaloid from plants; such as those from

Vinca species; bromine; compounds that contain bromine in their chemical structure; sodium azide; benzene. Suitable mutagenic conditions or physical mutagens are, e.g., UV irradiation, exposure to X-ray, and/or exposure to radioactivity.

The skilled person will appreciate that steps 2 to 4 above can be omitted, e.g., if the starting strain in step 1 is already sufficiently tolerant to acetic acid and/or propionic acid, or otherwise able to grow in a medium comprising stationary-phase supernatant of a propionibacterium cultivation. In this case the starting strain from step 1 is directly exposed to the mutagenic agent and/or condition in step 5.

A further aspect of the present invention relates to a fungal strain obtained by the above method.

### Brief description of the figures

Figure 1 shows the utilization of lactose by *Propionibacterium sp.* and the production of acetic and propionic acid and vitamin B12 in a two stage process on whey. In this way the COD value can be reduced from 85000 mg O<sub>2</sub>/L to 36000 mg O<sub>2</sub>/L. Up to 75 hours the fermentation was carried out without aeration and after 75 hours aeration was introduced.

Figure 2 shows the utilization of lactose by *Propionibacterium sp.* and the production of acetic and propionic acid and vitamin B12 in a two stage process on whey where tolerant DSM 28271 yeast culture was added in the stage where oxygen was introduced. In this way the COD value can be reduced from 85000 mg O<sub>2</sub>/L to 13000 mg O<sub>2</sub>/L. Up to 75 hours the fermentation was carried out without aeration and after 75 hours yeast and aeration was introduced.

Figure 3 shows a schematic presentation of a bioprocess for the co-cultivation of *Propionibacterium sp.* with tolerant yeast, according to the invention.

Figure 4 shows the utilization of lactose by *Propionibacterium sp.* and the production of acetic and propionic acid and vitamin B12 in a two stage process on whey where tolerant *K. lactis* ABLMKL6 yeast culture was added in the stage where oxygen was introduced. In this way the COD value can be reduced from 80000 mg O<sub>2</sub>/L to 14500 mg O<sub>2</sub>/L. Up to 112 hours the fermentation was carried out without aeration and after 112 hours yeast and aeration was introduced.

### Detailed description of the invention

The expression “supernatant”, or “culture supernatant”, in the context of the present invention, means the liquid obtained when filtrating or centrifuging a fermentation broth, thus removing cells and other insoluble material. The supernatant contains any nutrients and other components of the culture medium not (yet) consumed by the microorganism or degradation products, and any products produced by the microorganism during fermentation.

“Culture medium” shall be understood as being a nutrient-containing medium in which a microorganism can grow. Liquid culture media are preferred.

“Broth” or “fermentation broth” shall be understood as referring to a culture medium in which a microorganism grows, or has grown. A broth may comprise non-used nutrients and/or products produced by the microorganism.

A “stationary-phase supernatant” or “spent medium”, in accordance with the present invention shall be understood as being a supernatant from a stationary-phase fermentation broth.

The stationary phase shall be understood the phase of the cultivation in which the microorganism has substantially ceased to grow, e.g., the microorganism has reached its maximum cell density during the cultivation. The stationary phase of a cultivation can also be detected by monitoring the concentration of fermentation products. In one embodiment the stationary phase of a propionibacterium cultivation is defined as the phase starting from the point in time at which the concentration of acetic acid and/or propionic acid have reached its maximum value, or alternatively have reached 90% of its maximum value.

“Whey” is a by-product in the dairy industry, which separates from milk after curdling, when rennet or an acidic substance is added or formed *in situ*. “Sweet whey” is manufactured during the making of rennet types of hard cheese like cheddar or Swiss cheese. “Acid whey”, or “sour whey” is a by-product produced during the making of acid types of dairy products such as cottage cheese or strained yogurt.

The “chemical oxygen demand” or “COD” shall be understood as being the chemical oxygen demand as determined by ISO 6060:1989 standard method. It is understood that a sample may have to be diluted with water, if the COD to be determined is above the allowed maximum COD of 700 mg/L, according to this method. The COD is then calculated from the determined COD by multiplication with the dilution factor.

The present invention provides fungal cells, tolerant to co-cultivation with propionibacterium. This invention also provides a process of co-cultivating propionibacterium and a fungal cell, which cell is tolerant to inhibitory compounds produced by propionibacterium.

The invention provides fungal cells, which are capable of growing in the presence of propionibacterium and their inhibitory metabolites. These cells are obtained by procedures of selection of naturally occurring random mutants of generally available fungal cells on growth substrates in which propionibacterium has been previously cultivated. Preferably, selection of such tolerant fungal cells is carried out in at least two steps. In the first step random mutants of fungal cells are selected that can tolerate higher concentrations of organic acids, for example acetic acid and propionic acid. In the second step, random mutants of these acid-tolerant cells are subjected to additional round of selection on used growth medium in which propionibacterium has been previously cultivated and reached stationary phase of growth. Preferably the fungal cells, provided by this invention are yeast cells.



The fungal cells of this invention, which are tolerant to co-cultivation with propionibacterium, e.g., capable of growing in a stationary-phase supernatant of a propionibacterium culture, can be obtained by classical selection methods, possibly supported by random mutagenesis, by genetic engineering or by screening natural isolates.

5 A biotechnological process of the invention using tolerant yeast cells in a process of co-cultivation with propionibacterium may include the following steps:

1. Preparation of the culture medium
2. Inoculation with propionibacterium.
3. Fermentation without aeration
- 10 4. Switching to aerobic conditions
5. Inoculation with a tolerant yeast cell
6. Continuation of the fermentation as a co-cultivation of propionibacterium and yeast
7. Optionally downstream processing and product recovery

#### *The fermentation medium*

15 The fermentation medium can be any suitable fermentation medium in which both propionibacterium and fungal cells can grow. For example the fermentation medium may comprise molasses or whey. The fermentation medium may be composed of waste streams from different industries (such as whey) to which specific additives (such as minerals, vitamins, nitrogen and additional carbon sources, precursors etc.) may be added to increase  
20 the growth rate or the formation of desired products. The medium can contain different carbon sources such as glucose, lactose, fructose, lactic acid and nitrogen sources such as ammonium sulphate, amino acids, peptides and proteins that are suitable for propionibacterium. The pH value of the fermentation medium may be adjusted at the beginning of the process or can be maintained during the fermentation process to allow  
25 good growth of propionibacterium and/or product formation.

The medium is normally treated by a process to inactivate a sufficient proportion of microorganisms that would be initially present in the fermentation medium, before the inoculation with propionibacterium. These processes can be sterilization/pasteurization, e.g., autoclaving, filtration, irradiation and/or chemical treatments.

30 The cultivation vessel or fermenter should be prepared by a method that enables the removal of a sufficient proportion of microorganisms initially present and then filled with the fermentation medium. The cultivation vessel to be used in processes of the invention can be very simple, as long as it can maintain a desired temperature, and can maintain adequate stirring to prevent large pH or nutrient gradients. It can ideally withhold slight  
35 overpressure.

#### *Inoculation with Propionibacterium*

The inoculum with propionibacterium can consist of one or several stages depending on the final seed culture volume and the process used. The inoculum may be prepared in a medium that supports the growth of propionibacterium. The inoculum is cultivated at the

preferred temperature for the desired time after which it can be used to inoculate the fermentation medium. The inoculation volumes for subsequent stages of the inoculum or the fermentation stage can range from 1 to 20 %.

*Fermentation without aeration*

- 5 The fermentation broth is first maintained without aeration at the desired temperature for optimal growth or product formation. The temperature can be in the range of 25°C to 40°C, preferably at 35°C. If sugars are present in the fermentation medium the pH value should be maintained at the desired level, which can range from 5.5 to 8.0, preferably at 6.5. The pH can be maintained by several different acids/bases such as H<sub>2</sub>SO<sub>4</sub>, HCl, NaOH, NH<sub>4</sub>OH,  
10 etc. The bioprocess can be carried out without aeration or can be maintained under CO<sub>2</sub> and/or N<sub>2</sub> sparging and/or overpressure. Cultivation under CO<sub>2</sub> overpressure is favoured.

Stirring of the fermentation medium should be sufficient to prevent large pH gradients and can be performed but not limited by Rushton turbines, marine propellers or an internal and/or external recirculation pump.

15 *Switch to aerobic conditions*

After the nutrients consumable by propionibacterium are exhausted the culture can be switched to aerobic conditions. The air can be introduced into the cultivation vessel and should be sufficient for the growth of yeast described below. The aeration rate also influences the rate that yeast metabolises organic acids produced by propionibacterium.

- 20 Additional supplements may be introduced at this time that either influence the properties of the broth (i.e. antifoam), influence the formation of products produced by propionibacterium (i.e. 5,6-dimethylbenzimidazole) or influence the growth or product formation of yeast (nitrogen sources, precursors etc.).

- 25 After the culture has been switched to aerobic conditions the pH should again be maintained at the desired level. The pH value can range from 5.5 to 8, preferably at 6.5 to enable good growth of yeast.

*Inoculation of the propionibacterium-tolerant yeast cell*

- 30 The inoculum of the tolerant yeast cell can consist of one or several stages depending on the final volume of the seed culture. The yeast inoculum is prepared in a medium that supports the growth of yeast. The inoculum is cultivated at the preferred temperature for the desired time. The yeast inoculum can then be used to inoculate the fermentation medium with cultivated propionibacterium after it has been switched to aerobic conditions. The inoculation volumes for subsequent stages of the inoculum or the final stage can range but are not limited to 1 to 20 %. Preferably the yeast inoculum represents 5% of the final  
35 volume.

*Continuation of the fermentation by the resulting co-culture of microorganisms*

The fermentation broth is maintained at the desired temperature and the pH value is constantly maintained at the desired level by the addition of the appropriate acid or base. Stirring and aeration should be maintained at required levels to ensure complete utilization of organic acids. The amount of aeration and/or mixing influences the metabolism of organic acids by yeast.

Additional supplements can also be introduced at this time that either influence the properties of the broth (i.e. antifoam), influence the formation of products produced by propionibacterium or influence the growth or product formation of yeast. The COD value of the supernatant of the fermentation broth and yield of a valuable product, produced by either propionibacterium or yeast cells are monitored to achieve desired properties of the broth. Using such co-cultivation procedure the COD value of the supernatant of the broth can be decreased to less than 20000 mg O<sub>2</sub>/L, preferably to less than 15000 mg O<sub>2</sub>/L, more preferably to less than 10000 mg O<sub>2</sub>/L and even more preferably to less than 5000 mg O<sub>2</sub>/L. If the selected high-value product is vitamin B<sub>12</sub>, the yield of vitamin B<sub>12</sub> can be more than 5 mg/L, preferably more than 10 mg/L, more preferably more than 20 mg/L and even more preferably more than 100 mg/L.

*Downstream processing of the fermentation broth*

After the organic acids are consumed and COD of the supernatant reaches a satisfactory level, the bioprocess is stopped. Mixed propionibacterium/yeast biomass together with insoluble components of the broth can be separated from the supernatant by centrifugation, filtration or any other suitable method. The supernatant has low COD and presents a small burden if disposed to the water treatment plant or, in an ideal case, the COD is low enough that it can be disposed directly into the environment. If the biomass is enriched with valuable substances, such as vitamins and proteins, particularly vitamin B<sub>12</sub>, these substances can be used as an additive to animal feed. Alternatively, the biomass can be used as a starting material for isolation of valuable substances, such as vitamin B<sub>12</sub> in any form (e.g. cyanocobalamin or methylcobalamin). Depending on the grade of purity, vitamin B<sub>12</sub> can be used as an additive for animal feed or as a pharmaceutical or dietary supplement for human consumption.

The yeast according to the invention may be of the genus *Kluyveromyces* (e.g. *K. lactis*; *K. marxianus*), preferably *K. lactis*, and/or of the genus *Yarrowia*, preferably *Y. lipolytica*. However, the strain may also be of the genus *Debaryomyces* (e.g. *Debaryomyces hansenii*), *Candida* (e.g. *Candida versatilis*), *Cryptococcus*, *Rhodotorula*, *Pichia*, *Trichosporon* (e.g. *Trichosporon beigeli*), *Torulaspora*, *Issatchenkia* (e.g. *Issatchenkia orientalis*), *Geotrichum*, *Saccharomyces* or *Zygosaccharomyces*. Such cells are available in the art and can be either obtained from deposit institutions or they can be isolated from food products.

## Examples

### Example 1 – Preparation of propionibacterium-tolerant yeast cells

The yeast *Candida utilis* NRRL Y-7586 was cultivated in YEPD medium consisting of yeast extract (20 g/L), peptone (20 g/L) and dextrose (10 g/L) at 35°C for 72 hours, washed  
5 twice with 0.1M phosphate buffer (pH 7) and exposed to an adequate dose of an mutagenic agent (ethyl methanesulfonate) to obtain a 99.9% kill rate. Any other suitable fungal cell or yeast cell can be used. The surviving cells were cultivated in a medium containing yeast extract (10 g/L) and acetic and propionic acid at the minimal inhibitory concentration. After three days of cultivation at 35°C an aliquot from this broth was transferred to YEPD  
10 medium, left to grow for 72 hours and subjected to another round of mutagenesis. The surviving cells are then cultivated in a medium containing yeast extract (10 g/L) and an increased concentration of acetic and propionic acid (relative to the previous round). This procedure was iteratively repeated until the yeast was able to tolerate high concentrations of acetic and propionic acids (i.e. yeast able to grow and consume acetic and propionic  
15 acid in concentrations of 15 g/L). In this way the strain *C. utilis* ABLMCU1 was obtained.

With the resulting *C. utilis* strain (ABLMCU1), which was tolerant to high concentrations of acetic and propionic acids, a similar mutagenesis/selection scheme was used, this time using diluted stationary-phase supernatant from fermentation of propionibacterium as the inhibitory agent. In more detail, *C. utilis* cells, previously selected to be tolerant to organic  
20 acids, were grown in YEPD medium, subjected to ethyl methanesulfonate and subsequently cultivated on a medium which was a mixture of the stationary-phase supernatant obtained from fermentation of *Propionibacterium freudenreichii* strain ABLM1700 in whey (whey, 5 g/L yeast extract, 20 mg/L CoCl<sub>2</sub>, *Propionibacterium freudenreichii* cultivated for 96 hours at 35°C, supernatant hereinafter referred to as  
25 medium “As”) and the medium used for the development of yeast cells tolerant to organic acids (i.e. acetic acid (15 g/L), propionic acid (15 g/L) and yeast extract (10 g/L) (herein referred to as medium “Bs”). These media were mixed in a ratio of As:Bs = 3:7, which was above the level where ABLMCU1 yeast cells were able to grow. After 72 hours an aliquot of the resulting *C. utilis* culture was transferred to YEPD, grown and again exposed to  
30 ethyl methanesulfonate and subsequently transferred to the mixture of the media As and Bs at a higher ratio of As:Bs, namely 4:6. This procedure was repeated until yeast colonies were obtained, which were tolerant to undiluted stationary-phase supernatant of a fermentation of propionibacterium (100% As). The resulting yeast strain was deposited on 14 January 2014 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen  
35 (DSMZ) and is available under accession number DSM 28271.

The procedure described above resulted in a propionibacterium-tolerant *C. utilis* strain. It should be noted, however, that a similar procedure was used successfully to produce other propionibacterium-tolerant strains, i.e., starting from a different yeast strain. For example, *Kluyveromyces lactis* Y-17597 strain was treated in a similar procedure and resulted in  
40 propionibacterium-tolerant fungal strains (*K. lactis* ABLMKL6) according to the invention. The procedure was hence shown to be reproducible and applicable to other yeast strains.

*Example 2 –Test for establishing the “ability to grow in a stationary-phase supernatant of a propionibacterium cultivation”*

The following method is useful for determining whether a fungal stain, e.g. a yeast strain, is “capable of growing in a stationary-phase supernatant of a propionibacterium cultivation”, according to claim 1.

Step 1: Obtaining a stationary-phase supernatant of a propionibacterium cultivation

A first-stage propionibacterium seed culture is prepared in medium P1 (Table 1, below) as follows. 100 µL of propionibacterium stock culture, obtained from the culture collection (*Propionibacterium freudenreichii* ATCC 6207) is transferred to 50 mL of medium P1 and incubated for 4 days at 35°C without aeration and without shaking.

15 mL of this first-stage seed culture is then transferred to a 150 mL glass bottle filled with 135 mL of medium P2 (Table 2, below) and incubated for 4 days at 35°C without aeration and without shaking to obtain a second-stage seed culture.

100 mL of the so obtained second-stage seed culture is then used to inoculate a 1L working volume stirred tank bioreactor filled with 900 mL of medium P3 (Table 3, below). The cultivation parameters are: temperature:  $35 \pm 0.5$  °C, pH:  $6.5 \pm 0.1$  (controlled with 15% NaOH or H<sub>2</sub>SO<sub>4</sub>), agitation:  $100 \pm 10$  RPM, sparging with CO<sub>2</sub>:  $0.1 \pm 0.05$  vvm, NH<sub>4</sub><sup>+</sup> concentration:  $400 \pm 100$  mg/L (adjusted every 12 hours using 15% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, pH 6.5). The fermentation is run until the concentration of lactose is below 1g/L. At the end of the fermentation, the sum of the concentrations of acetic acid and propionic acid in the broth is preferably greater or equal to 20 g/L.

50 mL of the fermentation broth so obtained is centrifuged at 10000 g and the supernatant is transferred to an Erlenmeyer flask and autoclaved at 121°C for 20 minutes. The autoclaved medium is a “stationary-phase supernatant of a propionibacterium cultivation”.

Step 2: Testing the ability to grow in stationary phase supernatant of a propionibacterium

An inoculum of the fungal cell to be tested is prepared by adding 100 µL of a stock culture to 10 mL of Y1 medium (Table 4). The inoculum culture is incubated on a rotary shaker at 35°C and 200 RPM for 72 hours. The resulting culture is used as the “fungal inoculum” in the following steps.

The autoclaved Erlenmeyer flask containing the stationary-phase supernatant obtained in Step 1 above is inoculated with 2.5 mL of the fungal inoculum and incubated on a rotary shaker at 35°C and 200 RPM. The initial pH value is set to pH 6.5. This is considered the “test cultivation”.

Growth of the fungus is evaluated by measuring changes in the pH of the culture after 24 hours of cultivation. In one embodiment, a tested fungal cell is considered to be “capable of growing in a stationary-phase supernatant of a propionibacterium”, within the meaning of the appended claim 1, if the pH value of the broth in the test cultivation increases from the starting pH (pH 6.5) to a pH value of 8.0 or higher after 24 h of cultivation time.

Accordingly, the tested fungal cell may be regarded as *not* being capable of growing in a stationary-phase supernatant of a propionibacterium cultivation, if the pH value of the broth in the test cultivation remains below 8.0 after 24 h of cultivation time.

5 The claimed fungal cell being capable of growing in a stationary-phase supernatant of a propionibacterium cultivation is preferably one testing positive in the above test.

*Example 2 b) - Alternative Tests*

Alternatively, growth of the fungus can be evaluated by measuring changes OD of the culture after 24 hours of cultivation.

10 A tested fungal cell may be regarded as capable of growing in a stationary-phase supernatant of a propionibacterium cultivation, within the meaning of the appended claim 1, if the difference of the optical density (measured at 620 nm) of the broth is increased by at least 0.5 within 24 h after inoculation. Alternatively, a tested fungal cell is regarded as capable of growing in a stationary-phase supernatant of a propionibacterium cultivation, within the meaning of the appended claim 1, if the maximum growth rate ( $\mu_{\max}$ ) of the  
15 fungal cell in the test cultivation is equal to  $0.02 \text{ h}^{-1}$  or above.

Accordingly, a tested fungal cell is regarded as *not* being capable of growing in a stationary-phase supernatant of a propionibacterium cultivation, within the meaning of the appended claim 1, if the difference of the optical density (measured at 620 nm) of the broth is increased by less than 0.5 within 24 h after inoculation. Alternatively, a tested fungal cell  
20 is regarded as *not* being capable of growing in a stationary-phase supernatant of a propionibacterium cultivation, within the meaning of the appended claim 1, if the maximum growth rate ( $\mu_{\max}$ ) of the fungal cell in the test cultivation below  $0.02 \text{ h}^{-1}$ .

Accordingly, the claimed fungal cell being capable of growing in a stationary-phase supernatant of a propionibacterium cultivation is one testing positive in one of the above  
25 alternative tests.

Table 1: Defined medium P1 for propionibacterium

Ingredient	Amount
Trypticase (BBL)	10 g
Yeast extract (Difco)	10 g
Sodium DL-lactate(Sigma)	10 g
KH <sub>2</sub> PO <sub>4</sub> (Sigma)	2.5 g
MnSO <sub>4</sub> (Sigma)	0.05 g
Distilled water	up to 1000 mL
pH is adjusted to 7.0 with NaOH/HCl autoclave for 20 minutes at 121°C @ 1.2 bar	

Table 2: Defined medium P2 for propionibacterium

Ingredient	Amount
Glucose (Sigma)	40 g
Sodium DL-lactate (Sigma)	40 g
Yeast extract (Biolife)	10 g
CaCO <sub>3</sub> (Sigma)	10 g
CoCl <sub>2</sub> (Sigma)	10 mg
Calcium D-panthotenate* (Sigma)	20 mg
Distilled water	up to 1000 mL
pH is adjusted to 7.0 with NaOH/HCl autoclave for 20 minutes at 121°C @ 1.2 bar	

\*Added after sterilization

5 Table 3: Medium P3 for propionibacterium

Ingredient	Amount
Sweet whey powder**	60 g/L
Yeast extract (Biolife)	5 g/L
Calcium D-panthotenate* (Sigma)	40 mg/L
autoclave for 20 minutes at 121°C @ 1.2 bar	

\*Added after sterilization

\*\* Food-grade sweet whey powder with the following specifications: lactose min. 63%wt., protein min. 10%wt., moisture max. 5% is suitable and can be obtained from different suppliers such as: Hoogwegt International (Netherlands), Lactalis Ingredients (France), James Farrell & Co (USA).

10

Table 4: Medium Y1 for yeast

Ingredient	Amount
Bacto peptone (Biolife)	20 g
Yeast extract (Biolife)	10 g
Glucose (Sigma)	20 g
Distilled water	up to 1000 mL
autoclave for 20 minutes at 121°C @ 1.2 bar	

*Example 3 - Effective co-cultivation of propionibacterium and yeast*

## 15 i) Propionibacterium inoculum preparation

The inoculum of propionibacterium was prepared in two stages. A 0.5 mL stock suspension of *Propionibacterium freudenreichii* ABLM2475 (any other vitamin B12-producing strain, such as *Propionibacterium freudenreichii* ATCC6207, could have been used) was inoculated into 50 mL of the first vegetative medium (yeast extract 20 g/L and DL-lactate 20 g/L) and incubated for 4 days at 35°C. The first vegetative stage was then transferred to 400 mL of the second-stage vegetative medium (glucose 40 g/L, yeast extract 40 g/L, DL-lactate 40 g/L, calcium carbonate 10 g/L, cobalt chloride 20 mg/L, pantothenate 10 mg/L) and cultivated for 4 days while pH value being continuously neutralised with sodium hydroxide. The entire second stage was then transferred to the final bioreactor.

#### ii) Tolerant yeast inoculum preparation

The inoculum of tolerant yeast was also prepared in two stages. A 0.5 mL stock suspension of the yeast DSM 28271 was inoculated into 50 mL of the first vegetative medium (yeast extract 40 g/L, peptone 40 g/L and glucose 10 g/L) in a 250 mL Erlenmeyer flask and incubated for 2 days at 35°C on a rotary shaker at 220 RPM. The first vegetative stage was then transferred into 200 mL of the same medium in a 1000 mL Erlenmeyer flask and cultivated for 2 days at 35°C on a rotary shaker at 220 RPM.

#### iii) Fermentation

A 7 L working volume bioreactor was filled with 4 L of sour whey (COD was 80.000 mg O<sub>2</sub>/L) supplemented with yeast extract (5 g/L) and cobalt chloride (20 mg/L) and sterilized for 1 hour at 121°C. After cooling to 35°C pantothenate (10 mg/L) was added and the bioreactor inoculated with seed culture of propionibacterium. The cultivation temperature was 35°C and the agitation rate 100 RPM. The content of the bioreactor was sparged with CO<sub>2</sub> gas (10 mL/min) and the pH was maintained at 6.5 (with NaOH). After 90 hours the lactate and lactose have been exhausted and approximately 8 and 15 g/L acetic and propionic acid were produced. At this time 20 mg/L 5,6-dimethylbenzimidazole was added, the agitation rate was increased to 500 RPM and aeration introduced at 1 vvm and the bioreactor was inoculated with 5% of the tolerant yeast. The pH value was maintained at 6.5 (with H<sub>2</sub>SO<sub>4</sub>). After 48 hours all the organic acids were consumed by yeast and the fermentation was stopped. The process yielded 15 mg/L of vitamin B12. The biomass consisting of propionibacterium and yeast was removed by centrifugation and the COD value of the resulting supernatant was 12.000 mg O<sub>2</sub>/L, a 85 % reduction.

A schematic flow sheet of the exemplary process is shown in Figure 3.

#### Example 4 - Effective co-cultivation of propionibacterium and yeast (*K. lactis* ABLMKL6)

##### i) Propionibacterium inoculum preparation

The inoculum of propionibacterium was prepared in two stages. A 0.5 mL stock suspension of *Propionibacterium freudenreichii* ABLM2475 (any other vitamin B12-producing strain, such as *Propionibacterium freudenreichii* ATCC6207, could have been



used) was inoculated into 50 mL of the first vegetative medium (yeast extract 20 g/L and DL-lactate 20 g/L) and incubated for 4 days at 35°C. The first vegetative stage was then transferred to 400 mL of the second-stage vegetative medium (glucose 40 g/L, yeast extract 40 g/L, DL-lactate 40 g/L, calcium carbonate 10 g/L, cobalt chloride 20 mg/L, 5 pantothenate 10 mg/L) and cultivated for 4 days while pH value being continuously neutralised with sodium hydroxide. The entire second stage was then transferred to the final bioreactor.

ii) Tolerant yeast inoculum preparation

10 The inoculum of tolerant yeast was also prepared in two stages. A 0.5 mL stock suspension of the tolerant yeast *K. lactis* ABLMKL6 was inoculated into 50 mL of the first vegetative medium (yeast extract 40 g/L, peptone 40 g/L and glucose 10 g/L) in a 250 mL Erlenmeyer flask and incubated for 2 days at 35°C on a rotary shaker at 220 RPM. The first vegetative stage was then transferred into 200 mL of the same medium in a 1000 mL Erlenmeyer flask and cultivated for 2 days at 35°C on a rotary shaker at 220 RPM.

15 iii) Fermentation

A 7 L working volume bioreactor was filled with 4 L of sour whey (COD was 80.000 mg O<sub>2</sub>/L) supplemented with yeast extract (5 g/L) and cobalt chloride (20 mg/L) and sterilized for 1 hour at 121°C. After cooling to 35°C pantothenate (10 mg/L) was added and the bioreactor inoculated with seed culture of propionibacterium. The 20 cultivation temperature was 35°C and the agitation rate 100 RPM. The content of the bioreactor was sparged with CO<sub>2</sub> gas (10 mL/min) and the pH was maintained at 6.5 (with NaOH). After 112 hours the lactate and lactose have been exhausted and approximately 4 and 14 g/L acetic and propionic acid were produced. At this time 20 mg/L 5,6-dimethylbenzimidazole was added, the agitation rate was increased to 500 RPM and 25 aeration introduced at 1 vvm and the bioreactor was inoculated with 5% of the tolerant yeast. The pH value was maintained at 6.5 (with H<sub>2</sub>SO<sub>4</sub>). After 72 hours all the organic acids were consumed by yeast and the fermentation was stopped. The process yielded 16 mg/L of vitamin B12. The biomass consisting of propionibacterium and yeast was removed by centrifugation and the COD value of the resulting supernatant was 14.500 30 mg O<sub>2</sub>/L, a 81 % reduction.

**Claims**

1. A fungal cell capable of growing in a stationary-phase supernatant of a propionibacterium cultivation at a maximum growth rate  $\mu_{\max}$  of at least  $0.02 \text{ h}^{-1}$  wherein said stationary-phase supernatant is obtained from a stationary culture of  
5 *Propionibacterium freudenreichii* cultivated without aeration at  $35^{\circ}\text{C}$  in a liquid medium consisting of 60 g/L sweet whey powder, 5 g/L yeast extract, and 40 mg/L calcium D-pantothenate in water.
2. The fungal cell of claim 1, wherein the fungal cell is a yeast cell.
3. The yeast cell deposited on 14 January 2014 at the Deutsche Sammlung von  
10 Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, and available under accession number DSM 28271.
4. Process for producing a biotechnological product, said process comprising co-cultivation of propionibacterium and a fungal cell, said fungal cell being capable of growing in a stationary-phase supernatant of a propionibacterium cultivation, in a  
15 cultivation medium.
5. The process of claim 4, wherein said fungal cell is a yeast cell.
6. The process of claim 4, wherein said fungal cell is a fungal cell of any one of claims 1 to 3.
7. The process of any one of claims 4 to 6, wherein said biotechnological  
20 product is produced by said propionibacterium.
8. The process of any one of claims 4 to 7, wherein said biotechnological product is vitamin B12.
9. The process of any one of claims 4 to 8, wherein said process includes a phase without aeration followed by an aerobic phase.
10. The process of any one of claims 4 to 9, wherein at least 90% of the growth  
25 of the propionibacterium [in % gram dry weight] occurs during the phase without aeration.
11. The process of any one of claims 4 to 10, wherein at least 90% of the growth of the fungal cell [in % gram dry weight] occurs during the aerobic phase.
12. The process of any one of claims 4 to 11, wherein the chemical oxygen  
30 demand (COD) of the medium is reduced to a level of equal to or less than 25%, preferably equal to or less than 10%, of the initial COD of the cultivation medium.
13. The process of any one of claims 4 to 12, wherein said cultivation medium comprises whey.

\* \* \* \* \*

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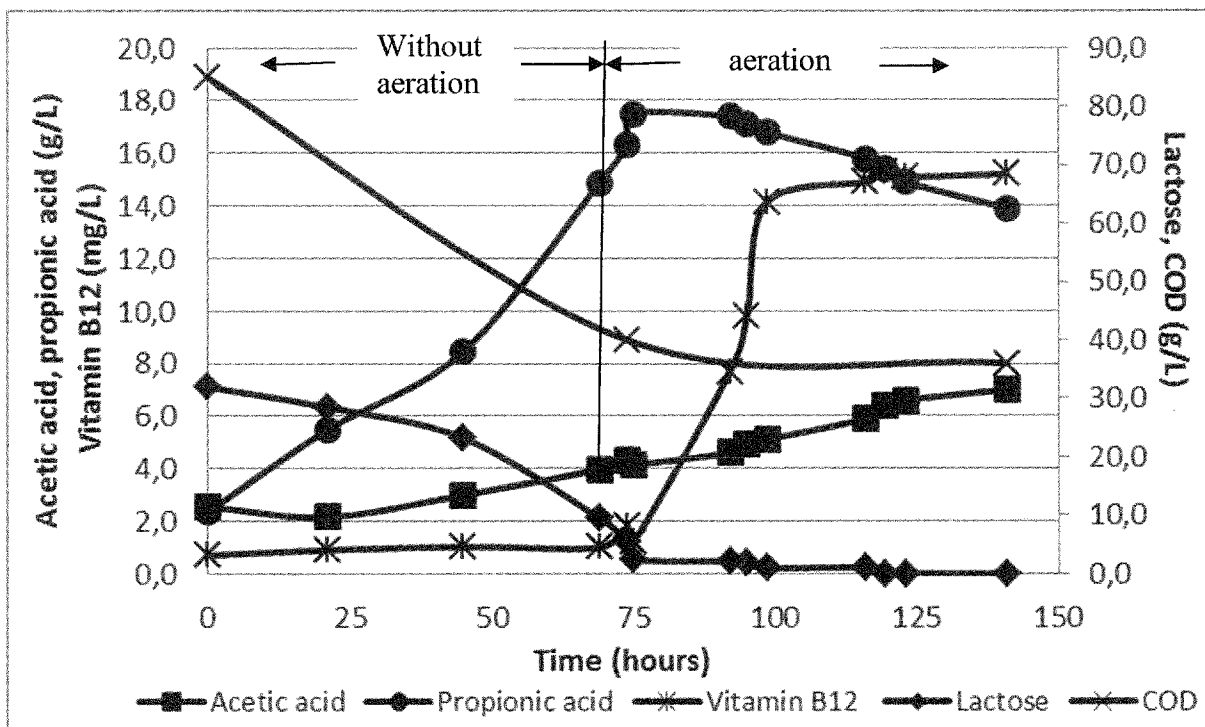


FIG. 1

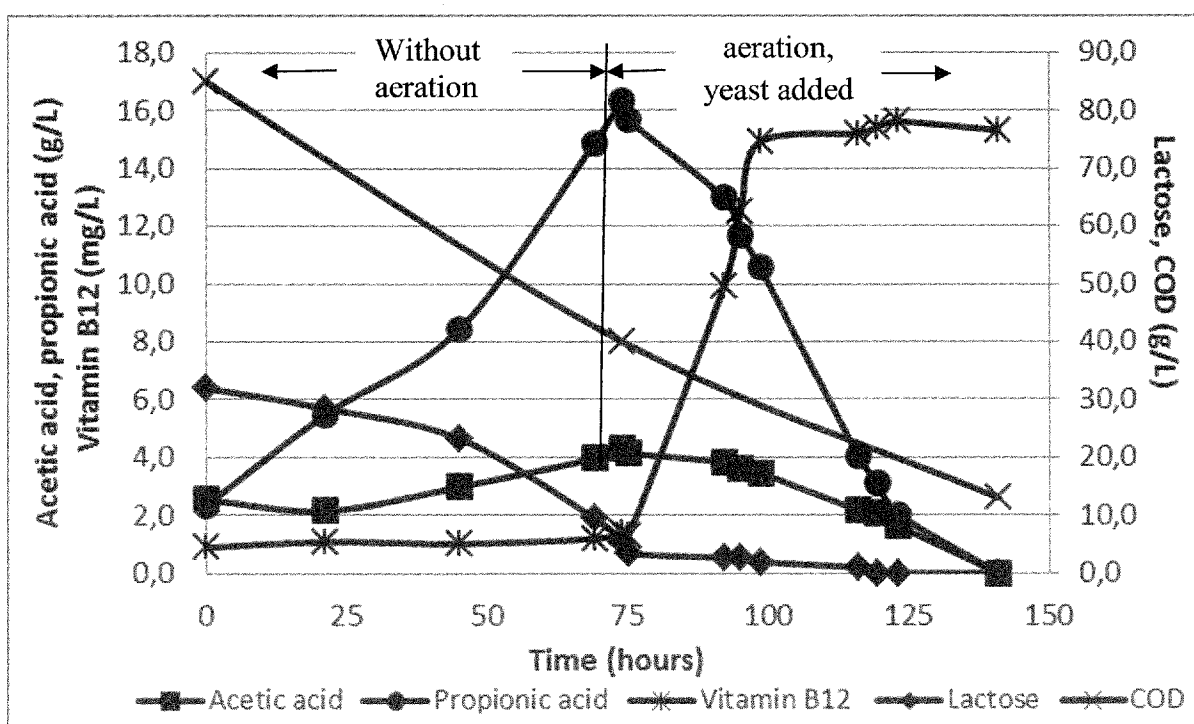


FIG. 2

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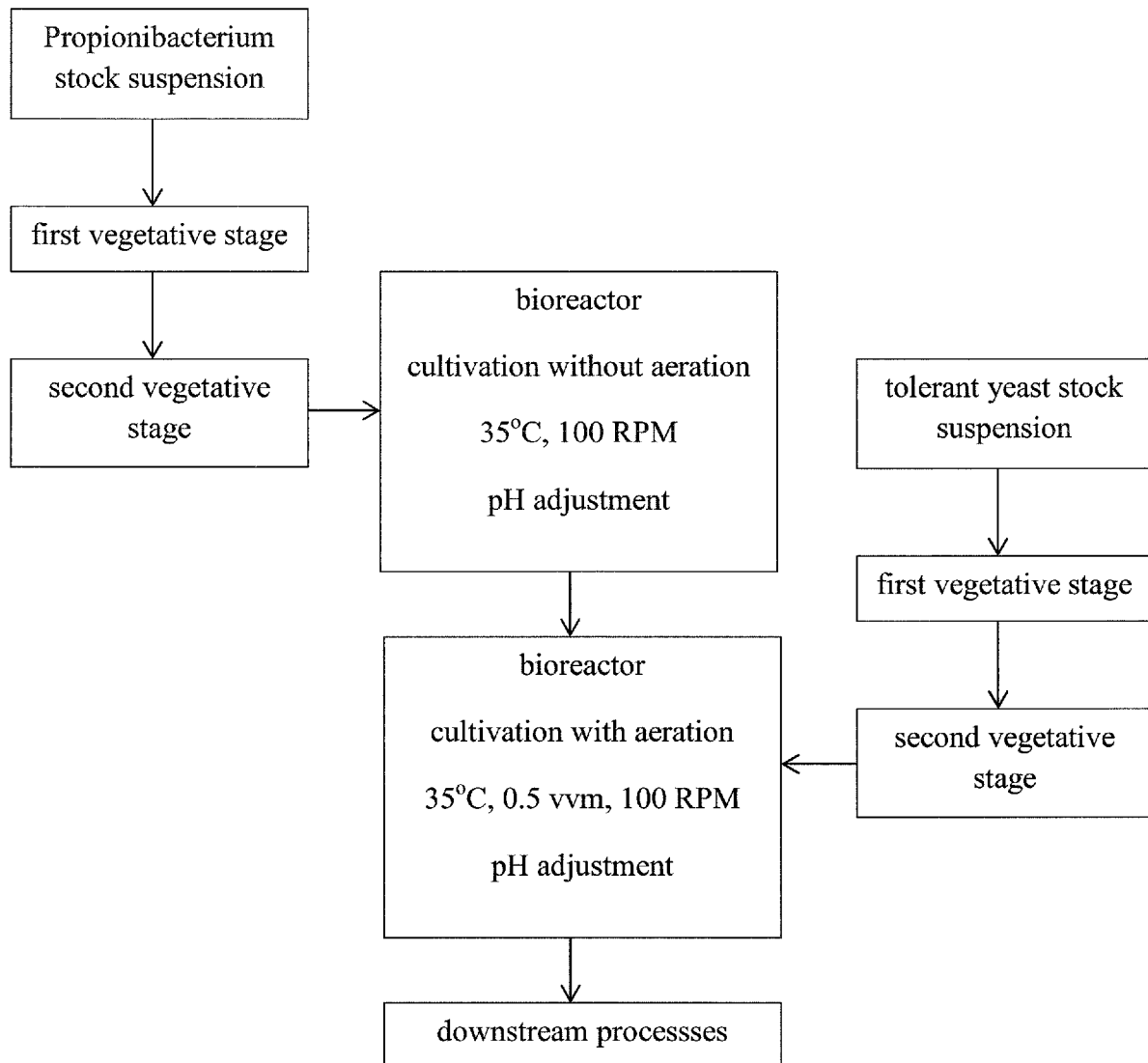


FIG. 3

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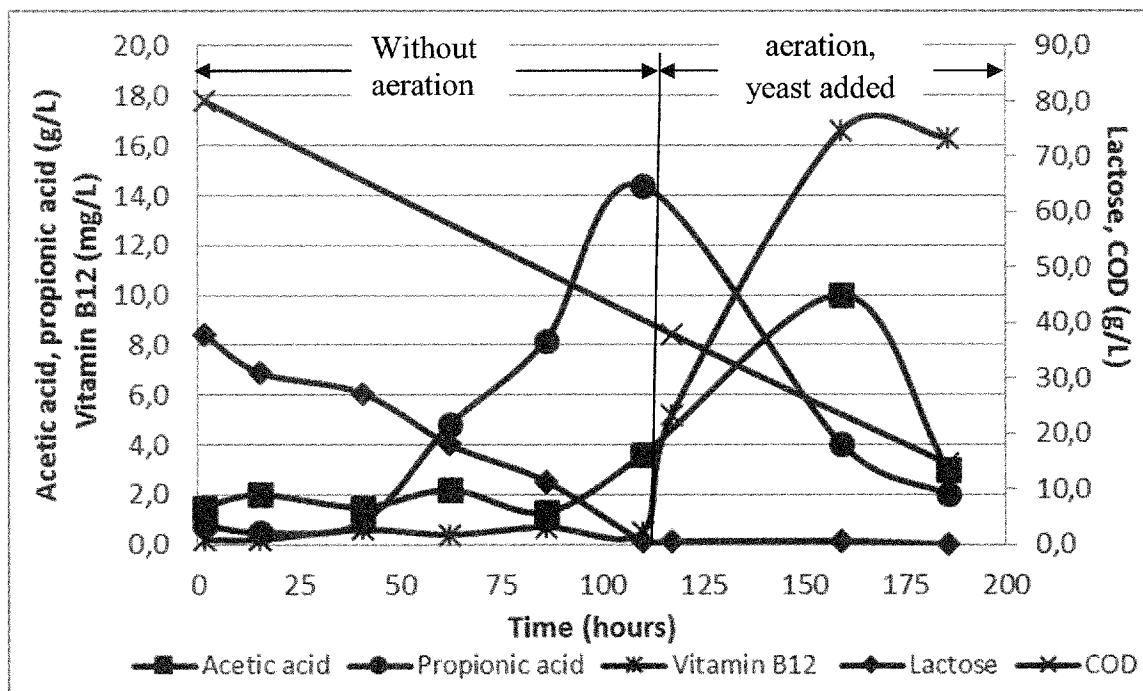


FIG. 4

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2015/060270

## A. CLASSIFICATION OF SUBJECT MATTER

INV. C12P39/00 C12R1/645 C12R1/01  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12P C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 2008/030089 A1 (CSK FOOD ENRICHMENT BV [NL]; BRANDSMA JOHANNES BERNHARD [NL]; MEIJER W) 13 March 2008 (2008-03-13) cited in the application the whole document ----- -/-	1,2,4-7



Further documents are listed in the continuation of Box C.



See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

1 September 2015

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2015/060270

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	----- WO 2011/140649 A1 (CORP HET HORIZON ENVIRONNEMENT TECHNOLOGIES [CA]; LEWANDOWSKI RAYMOND) 17 November 2011 (2011-11-17) cited in the application the whole document -----	1-13

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2015/060270

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