(19) Octrooicentrum Nederland

(11) **2023113**

(12) B1 **OCTROOI**

(21) Aanvraagnummer: **2023113**

(22) Aanvraag ingediend: **10 mei 2019**

(51) Int. Cl.:
C12P 7/56 (2019.01) **C12M 1/34** (2020.01)

(30) Voorrang:
-

(41) Aanvraag ingeschreven:
30 november 2020

(43) Aanvraag gepubliceerd:
-

(47) Octrooi verleend:
30 november 2020

(45) Octrooischrift uitgegeven:
7 december 2020

(73) Octrooihouder(s):
Technische Universiteit Delft te Delft

(72) Uitvinder(s):
Julius Laurens Rombouts te Delft
Marinus Cornelius Maria van Loosdrecht te Delft
Robbert Kleerebezem te Delft
David Gregory Weissbrodt te Delft
Gerben Roelandt Stouten te Delft
Maximilienne Toetie Allaart te Delft

(74) Gemachtigde:
mr. ir. J. van Breda c.s. te Amsterdam

(54) **High yield lactic acid production using mixed cultures**

(57) The present invention is in the field of a method of producing lactic acid in high yield in a sequencing batch reactor. Therein glucose may be used as feedstock for bacteria, which ferment the glucose into lactic acid. The reactor is operated under at least partly defined non-axenic conditions and in a cyclic mode.

NL B1 2023113

High yield lactic acid production using mixed cultures

FIELD OF THE INVENTION

The present invention is in the field of a method of producing lactic acid in high yield in a sequencing batch reactor. Therein glucose or other sugars may be used as feedstock for bacteria, which ferment the glucose into lactic acid. The reactor is at least partly operated under defined conditions and in a cyclic mode.

BACKGROUND OF THE INVENTION

Lactic acid ($CH_3CH(OH)CO_2H$) is a simple organic chemical compound that can be used in many applications. It is a chiral molecule and it occurs as L- or D-lactic acid. It is noted that lactic acid is highly soluble.

Lactic acid fermentation is performed on an industrial scale by lactic acid bacteria (*Lactobacillus species*), which convert simple carbohydrates such as glucose, sucrose, or galactose to lactic acid, or by chemical synthesis. Lactic acid producing bacteria can produce two moles of lactate from one mole of glucose, or one mole of lactate from one mole of glucose as well as carbon dioxide and acetic acid/ethanol, which latter is not preferred.

Typically, lactic acid fermentation is performed under rather strict conditions. First of all, a relatively pure culture is used, such as *Lactobacillus delbrueckii*. The aqueous solution in which fermentation is performed is typically partly neutralized, such as with lime. Typically, a pH of about 4.5-5.0 is used. The fermentation temperature is >50°C as production is often negligible up to 45°C. For fermentation typically a fermentor and seed reactor are used. In view of the pure culture sterilization of the feedstock and equipment used, e.g. the fermentor, is required. The yield of lactic acid is rather high, such as > 0.9 lactic acid/g glucose (yield on carbon of 90%), the productivity is good (e.g. >5 g/l*h) and the titer relatively high (>150 g/l lactic acid).

These prior art methods are however costly. A pure culture may attribute to some 15% of production costs. The inoculum costs some 3%, and energy consumption some 4%. The fermentor is relatively large (a few thousand cubic meter), the seed reactor

also has a significant volume (a few hundred cubic meters), and the need to be made from a relatively expensive material, such as stainless steel, making investments relatively high. As mentioned, proper sterilization is required, resulting in a down-time of the fermentor of some 20%, and high consumption of energy typically resulting in CO_2 production. In unlucky cases a phage infection may occur, resulting in the loss of a batch of feedstock and the need to obtain a phage resistant strain, which can delay the production process.

Lactic acid is a precursor for polylactic acid (PLA), which can be atactic or syndiotactic, and which polymers are biodegradable polyesters. Lactic acid may also be used as a food conservative, in cosmetics, and as a pharmaceutical component. The market size for lactic acid is huge (>5 M€/year) and growing. Therefore, there is a need for an efficient method of producing lactic acid.

The present invention therefore relates to an improved method of producing lactic acid by fermentation, which solves one or more of the above problems and drawbacks of the prior art, providing reliable results, without jeopardizing functionality and advantages.

SUMMARY OF THE INVENTION

It is an object of the invention to overcome one or more limitations of the methods and devices of the prior art and at the very least to provide an alternative thereto. In a first aspect the present invention relates to a method of producing lactic acid in a sequencing batch reactor comprising adaptively cycling (see e.g. fig. 1b) at least once between (i) a reaction phase, (ii) an effluent phase, and (iii) a feed phase, preferably 2-5000 times adaptive cycling, more preferably 4-1000, such as 5-1000 times. Each phase is considered to relate to a period of time. By coupling base dosing to substrate consumption and by closely monitoring a base consumption an adaptive cycle can be ended almost immediately when completed, consumption of substrate (e.g. glycose) is as fast as possible, resulting in high time-averaged volumetric rates. Typically, 1-20 adaptive cycles per day are possible, such as 2-10 adaptive cycles. Operation of the reactor can be continued for long periods of time. Maintenance can be performed 1-4 times per

year, or less. The present method uses a mixed culture capable of fermenting saccharide into lactic acid, which is added, such as to the reactor. Compared to prior art methods as described above the present method is considered to be >20% cheaper in terms of unit production costs of lactic acid. Some reasons thereto are that no sterilization is needed, no pure culture, such as a *Lactobacillus delbrueckii* culture, is required, no inoculum reactor and seed reactor are needed, the size of the present reactor can be a factor smaller and still having a similar production rate, the present method provides a high start biomass enabling a high productivity, such as > 6 g/l*h, the possibility of reusing biomass as protein and vitamin source therewith reducing peptide and vitamin source consumption, the (indirect) production of CO_2 is reduced significantly, scheduled down-time is low as no sterilization is required, and unscheduled down time, such as due to phage infection, is very unlikely due to the mixed culture comprising multiple strains and species. In addition, the throughput is higher. The down side is that somewhat lower yields (70% versus 90%) are obtained, which may be due to more biomass and possible byproduct, and in general there is no direct control over D/L ratio lactic acid; however, under specific conditions the latter can be resolved. Surprisingly at a pH of about 7 no detectable D-lactic acid (<0.1%) is formed and > 99.9% L-lactic acid.

The present method comprises a reaction phase wherein (ia1) maintaining the pH at a predetermined level between 5.6 and 8.5, preferably between 5.9 and 8.0, more preferably between 6.4 and 7.5, such as at 7.0, which is compared to prior art methods relatively high, (ia2) maintaining the temperature at 30-80 °C, preferably at 32-72 °C, more preferably at 35-63 °C, even more preferably at 37-60 °C, such as 38-50 °C, e.g. 40-45 °C, which preferred range is compared to prior art methods relatively low, (ia3) stirring the reaction phase, such as at 1-600 rpm, (ib) adding a base when the pH is below the predetermined level until the pH is at or above the predetermined level. The pH can be measured directly with a simple pH sensor. (ic1) Allowing fermentation to continue during a fermentation time until fermentation reaches a stationary phase, which stationary phase can be determined as then fermentation substantially stops, and

as a consequence the pH remains more or less constant, and no further base need to be added. So e.g. by monitoring an amount of base added over time, the stationary phase can be determined adequately, albeit with a small delay in time. (ic2) When fermentation has reached the stationary phase removing part of the effluent to the effluent phase, and (ic3) adding feed to the reaction phase, therewith largely or fully replenishing the reaction phase. The feed can be added subsequently to removing effluent from the reaction phase, during reaction, before reaction, and combinations thereof. In the effluent phase (iia) at least part of the lactic acid is removed, and (iib) preferably a remainder of the effluent phase is provided to the feed phase, preferably wherein 30-70% of broth is provided to the feed phase, such as 40-60% of broth. The lactic acid may be remover through an outlet towards a vessel or the like, it may be precipitated in the reactor, such as by adding Ca^{2+}, it may be separated over a membrane, and combinations thereof. So, a part of the broth may be recycled. In an alternative, or in addition, lactic acid may be removed from the reaction phase, such as by in-situ separation. In the feed phase (iiia) an aqueous feed mixture is provided comprising >10 g/l of a saccharide comprising compound, wherein the saccharide compound is selected from glucose, sucrose, fructose, galactose, lactose, disaccharides, oligo saccharides, poly saccharides, such as with 3-100 saccharides, starch, inulin, preferably wherein the saccharide comprising compound is a single compound, such as solely glucose, and >1 g peptide/100 g saccharide compound, or a combination thereof. It has been found that in addition to a saccharide as basic feed compound also peptides are required in order to obtain sufficient yield. Without the amount of peptide mentioned the yield drops to some 10%, much lower than the present 70% typically obtained. When starting the at least one adaptive cycle a mixed starting culture is added, which is capable of fermenting saccharide into lactic acid, such as added into the reactor.

In a second aspect the present invention relates to an apparatus for performing a method according to the invention, comprising a feed container, the feed container having a volume of 1-50 m^3, the feed container being in fluidic contact with a

reactor over a feed valve, the reactor having a pH-sensor in fluidic contact with the aqueous solution of the reactor, a reservoir comprising a base and a reservoir valve and optionally a pump, the reactor having a volume of 10-100 m^3, the reactor being in fluidic contact with an effluent container over an effluent valve, the effluent container having a volume of 1-50 m^3, and the effluent container being in fluidic contact with the feed container, each container and reactor having a mixer for rotating an aqueous phase with 1-600 rpm, the effluent container having an outlet for removing lactic acid, and the feed container having an input for replenishing feed, and a controller adapted (a) to open the reservoir valve when the pH drops below a predetermined level and to close said reservoir valve when the pH has reached said predetermined level again, (b) to open the feed valve when the fermentation reaches a stationary phase and to add feed, (c) to open the feed valve when the effluent has been partly removed and to add feed to replenish the reactor, and (d) to open the effluent valve when effluent is removed, such as when the liquid in the reactor has reached a predetermined volume.

The present invention provides a solution to one or more of the above-mentioned problems and overcomes drawbacks of the prior art.

Advantages of the present description are detailed throughout the description.

DETAILED DESCRIPTION OF THE INVENTION

In an exemplary embodiment of the present method biomass may be adaptive cycled at least once between the reaction phase, the effluent phase, and the feed phase. It has been found that biomass not only contributes to fermentation of saccharide into lactic acid, but also provides peptides, therewith improving the yield and throughput.

In an exemplary embodiment of the present method the feed phase may comprise > 80 g/l saccharide compound, such as 100-200 g/l.

In an exemplary embodiment of the present method the peptide concentration in the feed phase is between 1-40 g peptide/100 g saccharide, preferably between 2-30 g/100 g, more preferably between 5-15 g/100 g.

In an exemplary embodiment of the present method the peptide may be selected from monopeptides, dipeptides, tripeptides, tetrapeptides, biomass, and combinations thereof. In an example of biomass also peptides in yeast extract, or whey, or by-product of biological origin may be used. And further the biomass in the present system may be recycled, providing peptides, as well as vitamins or precursors thereof.

In an exemplary embodiment of the present method a reactor size may be 50-1000 m^3, such as 100-300 m^3. Such is much smaller than prior art reactors, therewith significantly reducing energy consumption in the production process, such as die to agitation (stirring) of the reaction phase.

In an exemplary embodiment of the present method the reactor may be a sequencing batch reactor or a sequencing fed batch rector. The present reactor may relate to a reactor comprising at least one of a zone of an apparatus, a phase of operation of an apparatus, and a sub-reactor. The sequencing in combination with adaptive cycling provides a quick start up, high throughput, and good adaptability of the system to potential varying characteristics.

In an exemplary embodiment of the present method no sterilization needs to be carried out.

In an exemplary embodiment of the present method no inoculation needs to be carried out.

In an exemplary embodiment of the present method no live yeast is present in the feed phase stock.

The above relate to a significant reduction in costs of operation, simplified mode of operation, reduced down-time, and reduced risk of infections.

In an exemplary embodiment of the present method the feed phase may comprise a vitamin B and/or precursor thereof, preferably 10^{-2}-10 mg vitamin B/g saccharide and/or precursor, preferably $2*10^{-2}$-5 mg/g vitamin B and/or precursor, such as 0.05-1 mg/g. It has been found that these vitamin and precursors improve the yield significantly.

In an exemplary embodiment of the present method the vitamin B may be selected from vitamin B_1 (thiamin), vitamin B_2 (riboflavin), vitamin B_3 (niacin, nicotinamide, nicotinamide riboside), vitamin B_5 (pantothenic acid), vitamin B_6

(pyridoxine), vitamin B_{12} (cobalamin), a salt thereof, such as a phosphate salt, and combinations thereof.

In an exemplary embodiment of the present method the precursor may be selected from precursors for coenzyme in catabolism of sugar, cofactor FAD, cofactor FMN, coenzyme NAD, coenzyme NADP, coenzyme A, a metabolic coenzyme, a fatty acid metabolism coenzyme, an amino acid metabolism coenzyme, and combinations thereof.

In an exemplary embodiment of the present method a culture titer of >40 g/l may be maintained, typically >50 g/l, such as > 100 g/l, i.e. high titers are obtainable.

In an exemplary embodiment of the present method a magnesium (cation) concentration in the feed phase may be 0.1-5 g/l, such as 0.2-2 g/l.

In an exemplary embodiment of the present method a calcium (cation) concentration in the feed phase may be >1.5 mg Ca/g saccharide. So, for 10-200 g saccharide/l more than 1.5-300 mg Ca/L may be provided.

The magnesium and calcium, typically provided as Mg^{2+} ion and Ca^{2+} ion, are found to contribute to fermentation.

In an exemplary embodiment of the present method the mixed culture may be enriched, such as by increasing an amount of saccharide compound, e.g. to >100 g/l, such as > 200g/l.

In an exemplary embodiment of the present method a biomass retention time may be controlled, such as between 1-8 days.

In an exemplary embodiment of the present method the reaction phase comprises >10% *Streptococcus*, such as > 50% *Streptococcus*, which can be determined by a semi-quantitative method like fluorescent in situ hybridization, or with any other method, such as with PCR.

In an exemplary embodiment of the present method a hydraulic retention time (HRT) may be from 1-8 days.

In an exemplary embodiment of the present method a base may be selected from hydroxides, oxides, and combinations thereof, preferably comprising Ca^{2+}, Na^{+}, or Mg^{2+}, and combinations thereof.

In an exemplary embodiment of the present method a pH may be maintained at 7.0±0.5, a temperature at 30-55 °C, a peptide amount at >2g/100g saccharide, a vitamin B at >0.1g/l, and

wherein >98% L-lactic acid is produced, such as > 99.9% L-lactic acid (on a mol/mol basis). This is rather surprising that virtually no D-lactic acid is produced, and substantially only L-lactic acid.

The invention will hereafter be further elucidated through the following examples which are exemplary and explanatory of nature and are not intended to be considered limiting of the invention. To the person skilled in the art it may be clear that many variants, being obvious or not, may be conceivable falling within the scope of protection, defined by the present claims.

SUMMARY OF THE FIGURES

Figs. 1a-b, 2, and 3a-b and 4 show some details of the present invention.

DETAILED DESCRIPTION OF FIGURES

In fig. 1a a schematic representation of the present adaptive cycle is shown. A feed phase is provided with a saccharide, and typically mixed. The feed is provided, in an adaptive cyclic mode, to a reaction phase, wherein microorganisms produce lactic acid from the saccharide. In the reaction phase the pH is measured. Upon decrease of the pH a source of base is activated, and base is added until the pH reaches a predetermined level. Then the addition of base is stopped, for the time being. The effluent of the reaction phase is transferred to an effluent phase, and the lactic acid is removed. A remainder of the effluent phase is fed back to the feed phase.

Initially base is added to the reaction phase, in order to compensate the pH for the lactic acid being formed. The amount of base over time decreases, until a plateau is reached. Then addition of base is stopped, as no further lactic acid is formed. The reactor volume as function of time/phase of operation is shown. Phases in a single adaptive cycle and theoretical associated base dosage profiles of the sequencing batch reactor used to perform adaptive base adaptive cycling. The adaptive cycle length was controlled by imposing a maximum base constant time, after which a new adaptive cycle was initiated, starting from the effluent phase.

Figure 2: Result of a FISH image using the str probe (light grey), specific for the streptococcus genus (Trebesius et

al. 2000) compared to the EUB338 probe mix (dark grey) (Amann et al. 1990).

Figs. 3a-b show microscopic images of bacterial cultures at pH=5 (left) and pH=7 (right), clearly showing that different bacteria are present at the higher pH.

Figure 4: Development of the read abundance of the 16S rRNA gene V3-V4 region in time on genus level. Various species are found, which develop over time in amount. Streptococcus, Enterobacteriaceae, Citrobacter, Klebsiella, and Clostridium are found in relatively high amount.

Examples

Materials and methods

Reactor operation

The enrichment was carried out in a 3 L bioreactor with a working volume of 2 L. Anaerobic conditions were maintained by continuously sparging the reactor with N_2 at a rate of 216 mL min^{-1} (@T=273K, P=10^5 kPa). The culture was taken out of the reactor for biofilm removal from reactor walls and head three times per week. The reactor was continuously agitated at a speed of 300 rpm using mechanical stirrers. Reactor temperature was maintained at 30°C by recirculating water heated at 31°C (E300 thermostat, Lauda, Germany) in the outer wall of the reactor. To prevent culture broth evaporation, the off-gas was cooled using a cryostat set to 5oC. Reactor pH was maintained at 5.0±0.2 using 8 mol L^{-1} NaOH and 1 mol L^{-1} HCl solutions (ADI 1030 Bio controller, Applikon, The Netherlands). To prevent excessive foaming, antifoaming agent (3% v:v antifoam C, Sigma Aldrich, Germany) was added in equal amounts and at equal speed as NaOH during 10 g L^{-1} glucose fermentations.

Enrichment medium

The enrichment at 10 g L^{-1} glucose was performed using a medium to which NH_4Cl, KH_2PO_4 and $FeCl_2.4H_2O$ were added to obtain a set molar C:N:P:Fe ratio of 100:5:1:0.33 and 1.5 g yeast extract was added per 10 g of glucose. Glucose and yeast extract solutions were autoclaved separately at 110°C for 20 minutes and then combined. Salts were supplied to the reactor from a second vessel, which was autoclaved at 121°C. Magnesium concentrations were adjusted to increasing glucose concentrations. Trace elements were supplied in sufficient amounts.

SBR phases and start-up

The reactor was operated in SBR mode. In a start-up phase for culture development the reactor was operated in batch mode until glucose was entirely consumed. For inoculation of the enrichment, 10 mL (0.5% v/v) of suspended and sieved (150 µm filtered) soil from the botanical garden of TU Delft was used (pH 7.4) and 10 mL of anaerobic digester sludge (Harnaschpolder, The Netherlands). After the start-up phase the SBR mode with an exchange ratio of 50% was entered. Three different SBR phases are distinguished: the effluent phase (5 minutes), the feed phase (4 minutes) and the reaction phase. The length of the reaction phase was dependent on the speed of microbial conversions in the reactor: adaptive base adaptive cycling was used to control the adaptive cycle time (Figure 1). The base constant time was initially kept at 90 minutes to avoid biomass washout, as an initial lag phase was observed in the adaptive cycles at the start of the enrichment. Throughout the enrichment, the base constant time was gradually decreased to 20 minutes.

Batch with 100 g L^{-1} glucose at pH 5

Anaerobicity, temperature, pH, agitation and broth evaporation were controlled as described for the sequencing batch reactors. After 2.5 days of operation the reactor was spiked with 0.25 times the initial amount of trace elements. Samples for monitoring biomass growth (OD660), glucose consumption and product formation were taken every 30 minutes for the first 2.5 hours of the cultivation. After this, samples were taken every hour until t = 8 h and finally 3 samples per day were taken. The final biomass concentration was calculated by measuring the volatile suspended solids (VSS) at the end of the fermentation.

SBR operation at pH 7

The enrichment at pH 7 was carried out as described for pH 5, but the pH was controlled at 7.2±0.2 using 8 mol L-1 NaOH. The reactor was inoculated with 5 mL suspended and sieved soil and 5 mL anaerobic digester sludge. Adaptive base adaptive cycling with a minimal base constant time of 10 minutes was used for the first two weeks of enrichment, after which a fixed adaptive cycle length of 90 minutes was set. Initially, the culture broth

was agitated at 300 rpm. However, after 145 SRTs the stirring speed was increased to 600 rpm to improve mass transfer of carbon dioxide to the gas phase.

Batch with 100 g L^{-1} glucose at pH 7

The batch reactor was operated as described for the batch process at pH 5. The reactor was inoculated with cell pellets of approximately 1L of the culture obtained at the end of the enrichment at pH 7. Samples were taken every 30 minutes for the first 3.5 hours and every hour until glucose was nearly depleted (<16 mM residual glucose). Antifoam was added manually when foaming occurred. Culture was stored in the fridge overnight before collecting the pellet for VSS determination.

Analytical methods

The gas productivities (H_2 and CO_2) and acid/base dosages for pH control were monitored on-line using MFCS software (Sartorius, Germany) and a Rosemount Analytical NGA 2000 (Emerson, USA). Biomass concentrations were monitored both by measuring optical density (OD660) and the amount of VSS in the broth as described earlier using 150 mL effluent (APHA/AWWA/WEF 1999), calculated assuming a biomass molecular weight of 24.6 g mol^{-1}. Both OD and VSS were always determined in duplicate. Glucose, ethanol and VFA concentrations were determined using high performance liquid chromatography (HPLC) using an Aminex HPX-87H column (BioRad, USA) at 59°C coupled to a refractive index- and ultraviolet detector (Waters, USA). 1.5 mmol L^{-1} phosphoric acid was used as eluent. Biomass was removed from the reactor samples by centrifugation and filtration using a 0.22 µm membrane filter (Millipore, Millex-GV, Ireland).

Microbial community analysis

DNA was extracted from cell pellets from different time points in the enrichment using the DNAeasy microbial extraction kit (Qiagen Inc., Germany) following manufacturer's instructions and sent to Novogene (Hong Kong, China) for 16S rRNA amplicon sequencing of the V3-V4 region as described by Rombouts et al., (2019).

Fluorescent in situ hybridization (FISH) was used for analyzing the microbial community with epifluorescence microscopy (Axioplan 2 imaging, Zeiss, Germany). Fixation and overnight hybridization were performed as described by Johnson

et al. (2009) using the probes listed in table 1. An additional DAPI staining targeting all microbial cells was used by incubating the slides for 15 minutes with 10 µL of 10 mg mL^{-1} DAPI solution after washing and drying.

Microorganism	Probe	Forma-mide (%)	Sequence (5′ → 3′)
Eubacteria	EUB338	5-30	GCTGCCTCCCGTAGGAGT
Lactobacillus	Lacto772	25	YCACCGCTACACATGRAGTTCCACT
Lactococcus	Lactococcus4	5	CTGTATCCCGTGTCCCGAAG
Megasphaera	Mega-X	25	GACTCTGTTTTTGGGGTTT
Streptococcus	Str	30	CAC TCT CCC CTT CTG CAC
Enterobacteriaceae	Ent183	20	CTC TTT GGT CTT GCG ACG

Results

A product spectrum was monitored in time of both enrichments. Lactate was the main fermentation product in both conditions. At 30 SRTs, the amount of trace elements was doubled, leading to mainly lactate production. At pH 7, a 5.4 times higher productivity was reached. What is very surprising is that only L-lactic acid is produced at pH 7, while a nearly racemic mixture is produced at pH 5.

		pH 5	pH 7
10 g L^{-1} glucose	*Maximum obtained lactate yield (g_p g_s^{-1})*	0.76	0.69
	Maximum obtained productivity (g L^{-1} h^{-1})	1.16	6.24
	Ratio D:L – lactate	53:47	0:100
	$Y_{x/s}$ ($Cmol_x$ $Cmol_s^{-1}$)	0.14	0.20
	q_s ($Cmol_s$ $Cmol_x^{-1}$ h^{-1})	0.74	2.25
	$\mu_{average}$ (h^{-1})	0.11	0.46
	$Y_{LA/s}$ (g_p g_s^{-1})	0.60	0.59
100 g L^{-1} glucose			
	Final attained lactate concentration (g L^{-1})	57.6	56.6
	$Y_{x/s}$ ($Cmol_x$ $Cmol_s^{-1}$)	0.09	0.14
	Average productivity (g L^{-1} h^{-1})	0.73	4.72

The microbial community analysis revealed that the pH enrichment was predominated by *Lactobacillus* species. A significant side population of *Megasphaera* was detected. At pH 7, *Streptococcus* was observed to be predominant genus, with also several *Enterobacteriaceae* genera occurring, such as *Klebsiella* and *Citrobacter*. The amount of Streptococcus was shown to be very dominant, in the range of >90% of the biomass.

Conclusions

Obtaining selective L-lactic acid production using mixed cultures without sterilisation and a pure culture inoculum was achieved. An acidic environment at pH 5 was tested in comparison to a neutral pH environment at pH 7 under the same cultivation conditions. A complex medium was used, as this stimulates the growth of lactic acid bacteria. The adaptive cycle times could be adjusted to the time base dosage stopped and the substrate, glucose, was assumed to be depleted (the 'plateau' was reached). It was found that lactic acid bacteria could be successfully enriched at both pH 5 and pH 7 using the described enrichment strategy. Further, lactic acid production at pH 7 is favoured over pH 5, as the productivity is higher and there is selective production of L-lactic acid.

Inventors have obtained a high yield of lactic acid on glucose using a defined medium and defined bioreactor conditions. A stable yield of >70% LA g/g of glucose, and a productivity of 6.2 g/l*h in a 2l bioreactor at 10 g/l glucose and a titer of 57 g/l is obtained in the reactor. These conditions, or more explicitly, these ecological parameters, can be applied to optimize current large-scale fermentations producing lactic acid. Also, current waste streams can be used to ferment these to lactic acid at high yield and refine the lactic acid out of the stream.

With the present method and system, a maximum obtained yield of 0.69(molp $mols^{-1}$), a maximum obtained productivity of 6.24(g L^{-1} h^{-1}), a ratio D:L-lactate of 0-100, and a final attained lactate concentration of 56.6 (g L^{-1}) were obtained.

For the sake of searching the following section is added which represents a translation of the subsequent section.

1. Method of producing lactic acid in a sequencing batch reactor comprising

adaptively cycling at least once between (i) a reaction phase, (ii) an effluent phase, and (iii) a feed phase, preferably 2-5000 times cycling,

in the reaction phase

(ia1) maintaining the pH at a predetermined level between 5.6 and 8.5, preferably between 5.9 and 8.0, more preferably between 6.4 and 7.5, such as at 7.0,

(ia2) maintaining the temperature at 30-80 °C, preferably at 35-72 °C, more preferably at 37-63 °C, such as 40-45 °C,

(ia3) stirring the reaction phase, such as at 1-600 rpm,

(ib) adding a base when the pH is below the predetermined level until the pH is at or above the predetermined level,

(ic1) allowing fermentation to continue during a fermentation time until fermentation reaches a stationary phase,

(ic2) when fermentation has reached the stationary phase removing part of the effluent to the effluent phase, and

(ic3) adding feed to the reaction phase,

in the effluent phase

(iia) removing at least part of the lactic acid, and

(iib) preferably providing a remainder of the effluent phase to the feed phase, preferably wherein 30-70% of broth is provided to the feed phase, such as 40-60% of broth,

in the feed phase

(iiia) providing an aqueous feed mixture comprising

>10 g/l of a saccharide comprising compound, wherein the saccharide compound is selected from glucose, sucrose, fructose, galactose, lactose, disaccharides, oligo saccharides, poly saccharides, starch, inulin, preferably a single compound, or a combination thereof, and

>1 g peptide/100 g saccharide compound, and

adding a mixed starting culture capable of fermenting saccharide into lactic acid, such as into the reactor.

2. Method according to embodiment 1, wherein biomass is cycled at least once between the reaction phase, the effluent phase, and the feed phase.

3. Method according to any of embodiments 1-2, wherein the feed

phase comprises > 80 g/l saccharide compound, such as 100-200 g/l.

4. Method according to any of embodiments 1-3, wherein the peptide concentration in the feed phase is between 1-40 g peptide/100 g saccharide, preferably between 2-30 g/100 g, more preferably between 5-15 g/100 g.

5. Method according to any of embodiments 1-4, wherein the peptide is selected from monopeptides, dipeptides, tripeptides, tetrapeptides, biomass, and combinations thereof.

6. Method according to any of embodiments 1-5, wherein a reactor size is 50-1000 m^3.

7. Method according to any of embodiments 1-5, wherein the reactor is a sequencing batch reactor or a sequencing fed batch rector.

8. Method according to any of embodiments 1-7, wherein no sterilization and/or no inoculation is carried out and/or no live yeast is present in the feed phase stock.

9. Method according to any of embodiments 1-8, wherein the feed phase comprises vitamin B and/or precursor thereof, preferably 10^{-2}-10 mg vitamin B/g saccharide and/or precursor.

10. Method according to embodiment 9, wherein the vitamin B is selected from vitamin B_1 (thiamin), vitamin B_2 (riboflavin), vitamin B_3 (niacin, nicotinamide, nicotinamide riboside), vitamin B_5 (pantothenic acid), vitamin B6 (pyridoxine), vitamin B_{12} (cobalamin), a salt of said vitamins, and combinations thereof.

11. Method according to embodiment 9, wherein the precursor is selected from precursors for coenzyme in catabolism of sugar, cofactor FAD, cofactor FMN, coenzyme NAD, coenzyme NADP, coenzyme A, a metabolic coenzyme, a fatty acid metabolism coenzyme, an amino acid metabolism coenzyme, and combinations thereof.

12. Method according to any of embodiments 1-11, wherein a culture titer of >40 g/l is maintained, such as >50 g/l.

13. Method according to any of embodiments 1-11, wherein a magnesium (cation) concentration in the feed phase is 0.1-5 g/l, and/or wherein a calcium (cation) concentration in the feed phase is >1.5 mg Ca/g saccharide.

14. Method according to any of embodiments 1-13, wherein the mixed culture is enriched.

15. Method according to any of embodiments 1-14, wherein a biomass retention time is controlled, such as between 1-8 days.

16. Method according to any of embodiments 1-15, wherein the reaction phase comprises >10% *Streptococci*.

17. Method according to any of embodiments 1-16, wherein a hydraulic retention time (HRT) is from 1-8 days.

18. Method according to any of embodiments 1-17, wherein a base is selected from hydroxides, oxides, and combinations thereof, preferably comprising Ca^{2+}, Na^{+}, or Mg^{2+}, and combinations thereof.

19. Method according to any of embodiments 1-18, wherein a pH is maintained at 7.0±0.5, a temperature at 30-55 °C, a peptide amount at >2g/100g saccharide, a vitamin B at >0.1g/l, and wherein >98% L-lactic acid is produced, such as > 99.9% L-lactic acid (on a mol/mol basis).

20. Apparatus for performing a method according to any of embodiments 1-19, comprising a feed container, the feed container having a volume of 1-50 m^3, the feed container being in fluidic contact with a reactor over a feed valve, the reactor having a pH-sensor in fluidic contact with the aqueous solution of the reactor, a reservoir comprising a base and a reservoir valve, the reactor having a volume of 10-100 m^3, the reactor being in fluidic contact with an effluent container over an effluent valve, the effluent container having a volume of 1-50 m^3, and the effluent container being in fluidic contact with the feed container, each container and reactor having a mixer for rotating an aqueous phase with 1-600 rpm, the effluent container having an outlet for removing lactic acid, and the feed container having an input for replenishing feed, and

a controller adapted (a) to open the reservoir valve when the pH drops below a predetermined level and to close said reservoir valve when the pH has reached said predetermined level again,

(b) to open the feed valve when the fermentation reaches a stationary phase and to add feed,

(c) to open the feed valve when the effluent has been partly removed and to add feed to replenish the reactor, and

(d) to open the effluent valve when effluent is removed, such as when the liquid in the reactor has reached a predetermined volume.

CONCLUSIES

1. Werkwijze voor het produceren van melkzuur in een sequentie-batchreactor omvattende
het ten minste één keer adaptief doorlopen van een kringloop tussen (i) een reactiefase, (ii) een effluentfase, en (iii) een toevoerfase, bij voorkeur 2-5000 maal doorlopen van de kringloop,
in de reactiefase
(ia1) het handhaven van de pH op een vooraf bepaald niveau tussen 5,6 en 8,5, bij voorkeur tussen 5,9 en 8,0, liever tussen 6,4 en 7,5, zoals op 7,0,
(ia2) het handhaven van de temperatuur op 30-80 °C, bij voorkeur op 35-72 °C, liever op 37-63 °C, zoals 40-45 °C,
(ia3) het roeren van de reactiefase, zoals bij 1-600 rpm,
(ib) het toevoegen van een base wanneer de pH onder het vooraf bepaalde niveau is totdat de pH op of boven het vooraf bepaalde niveau is,
(ic1) het fermenteren laten doorgaan gedurende een fermentatietijd totdat de fermentatie een stationaire fase bereikt,
(ic2) wanneer de fermentatie de stationaire fase heeft bereikt het verwijderen van een deel van het effluent naar de effluentfase, en
(ic3) het toevoeren van voeding aan de reactiefase,
in de effluentfase
(iia) het verwijderen van ten minste een deel van het melkzuur, en
(iib) bij voorkeur het verschaffen van een rest van de effluentfase aan de toevoerfase, bij voorkeur waarbij 30-70% kweek wordt verschaft aan de toevoerfase, zoals 40-60% kweek,
in de toevoerfase
(iiia) het verschaffen van een waterig voedingsmengsel omvattende
> 10 g/l van een sacharide omvattende verbinding, waarbij de sacharideverbinding is gekozen uit glucose, sucrose, fructose, galactose, lactose, disachariden,

oligosachariden, polysachariden, zetmeel, inuline, bij voorkeur een enkele verbinding, of een combinatie daarvan, en

> 1 g peptide/100 g sacharideverbinding, en

het toevoegen van een gemengde uitgangscultuur die sacharide tot melkzuur kan fermenteren, zoals in de reactor.

2. Werkwijze volgens conclusie 1, waarbij de biomassa ten minste eenmaal wordt rondgevoerd tussen de reactiefase, de effluentfase en de toevoerfase.

3. Werkwijze volgens een der conclusies 1-2, waarbij de toevoerfase> 80 g/l sacharideverbinding omvat, zoals 100-200 g/l.

4. Werkwijze volgens een van de conclusies 1-3, waarbij de peptideconcentratie in de voedingsfase tussen 1-40 g peptide/100 g sacharide is, bij voorkeur tussen 2-30 g/100 g, liever tussen 5-15 g/100 g.

5. Werkwijze volgens een van de conclusies 1-4, waarbij de peptide is gekozen uit monopeptiden, dipeptiden, tripeptiden, tetrapeptiden, biomassa, en combinaties daarvan.

6. Werkwijze volgens een van de conclusies 1-5, waarbij een reactorafmeting 50-1000 m^3 is.

7. Werkwijze volgens een van de conclusies 1-5, waarbij de reactor een sequencing batchreactor is of een sequentie-gevoede batch-rector.

8. Werkwijze volgens een van de conclusies 1-7, waarbij geen sterilisatie en/of geen inoculatie wordt uitgevoerd en/of geen levende gist aanwezig is in de voorraad van de voedingsfase.

9. Werkwijze volgens een van de conclusies 1-8, waarbij de toevoerfase vitamine B en/of precursor daarvan omvat, bij voorkeur 10^{-2}-10 mg vitamine B/g sacharide en/of voorloper omvat.

10. Werkwijze volgens conclusie 9, waarbij de vitamine B is gekozen uit vitamine B_1 (thiamine), vitamine B_2 (riboflavine), vitamine B_3 (niacine, nicotinamide, nicotinamide riboside), vitamine B_5 (pantotheenzuur), vitamine B_6 (pyridoxine) vitamine B_{12} (cobalamine), een zout van genoemde vitaminen, en combinaties daarvan.

11. Werkwijze volgens conclusie 9, waarbij de precursor is gekozen uit voorlopers voor co-enzym in katabolisme van suiker, cofactor FAD, cofactor FMN, co-enzym NAD, co-enzym NADP, co-

enzym A, een metabolisch co-enzym, een vetzuurmetabolisme co-enzym, een aminozuur metabolisme co-enzym, en combinaties daarvan.

12. Werkwijze volgens een van de conclusies 1-11, waarbij een kweektiter van> 40 g/l wordt gehandhaafd, zoals> 50 g/l.

13. Werkwijze volgens een van de conclusies 1-11, waarbij een magnesium kation concentratie in de toevoerfase 0,1-5 g/l is, en/of waarbij een calcium kation concentratie in de toevoerfase> 1,5 mg Ca is/g sacharide.

14. Werkwijze volgens een van de conclusies 1-13, waarbij de gemengde cultuur is verrijkt.

15. Werkwijze volgens een van de conclusies 1-14, waarbij een retentietijd van biomassa wordt geregel, zoals tussen 1-8 dagen.

16. Werkwijze volgens een der conclusies 1-15, waarbij de reactiefase >10% streptokokken omvat.

17. Werkwijze volgens een van de conclusies 1-16, waarbij een hydraulische retentietijd (HRT) 1-8 dagen is.

18. Werkwijze volgens een van de conclusies 1-17, waarbij een base is gekozen uit hydroxiden, oxiden, en combinaties daarvan, bij voorkeur omvattende Ca^{2+}, Na^{+} of Mg^{2+}, en combinaties daarvan.

19. Werkwijze volgens een van de conclusies 1-18, waarbij een pH wordt gehandhaafd op 7,0±0,5, een temperatuur op 30-55 °C, een hoeveelheid peptide op >2 g/100 g sacharide, een vitamine B op >0,1 g/l, en waarbij >98% L-melkzuur wordt geproduceerd, zoals >99,9% L-melkzuur (op een mol/mol-basis).

20. Inrichting voor het uitvoeren van een werkwijze volgens één van de conclusies 1-19, omvattende een toevoerhouder, waarbij de toevoerhouder een volume heeft van 1-50 m^3, waarbij de toevoerhouder in fluïdumcontact staat met een reactor over een toevoerklep, de reactor een pH-sensor heeft in vloeibaar contact met de waterige oplossing van de reactor, een reservoir dat een base en een reservoirklep omvat, waarbij de reactor een volume heeft van 10-100 m^3, waarbij de reactor in fluïdumcontact staat met een effluenthouder over een effluentklep, waarbij de effluenthouder een volume heeft van 1-50 m^3, en waarbij de effluenthouder in fluïdumcontact staat met de toevoerhouder, waarbij elke houder en reactor een menger heeft voor het roteren

van een waterige fase met 1-600 rpm, waarbij de effluenthouder een uitlaat voor het verwijderen van melkzuur heeft, en de toevoerhouder een invoer heeft voor het aanvullen van voeding, en

een regelaar aangepast (a) om de reservoirklep te openen wanneer de pH onder een vooraf bepaald niveau daalt en om de reservoirklep te sluiten wanneer de pH het vooraf bepaalde niveau opnieuw heeft bereikt,

(b) om de toevoerklep te openen wanneer de fermentatie een stationaire fase bereikt en om voeding toe te voegen,

(c) om de toevoerklep te openen wanneer het effluent gedeeltelijk is verwijderd en om voeding toe te voegen om de reactor aan te vullen, en

(d) om de effluentklep te openen wanneer effluent wordt verwijderd, zoals wanneer de vloeistof in de reactor een vooraf bepaald volume heeft bereikt.

Fig. 1a

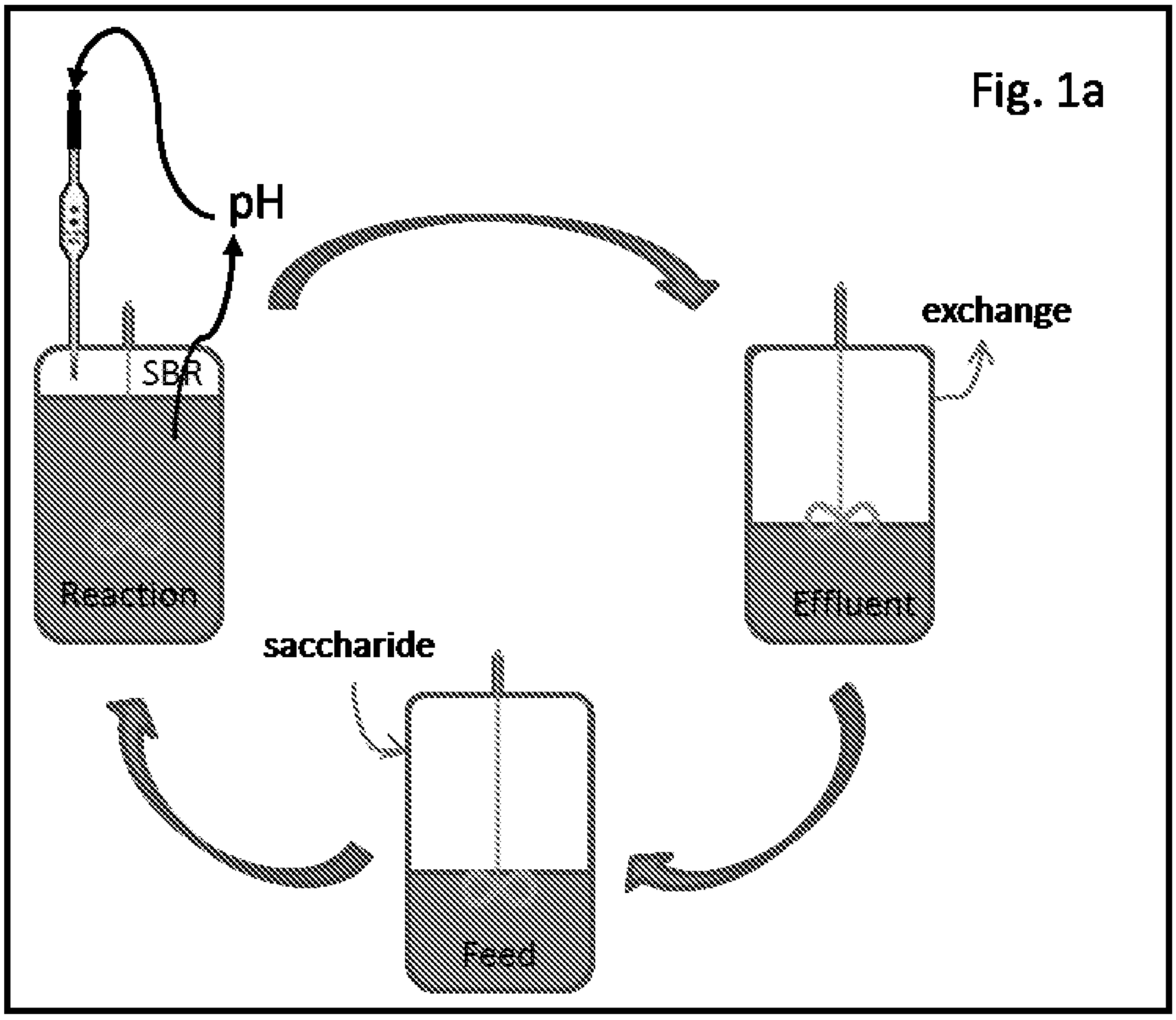
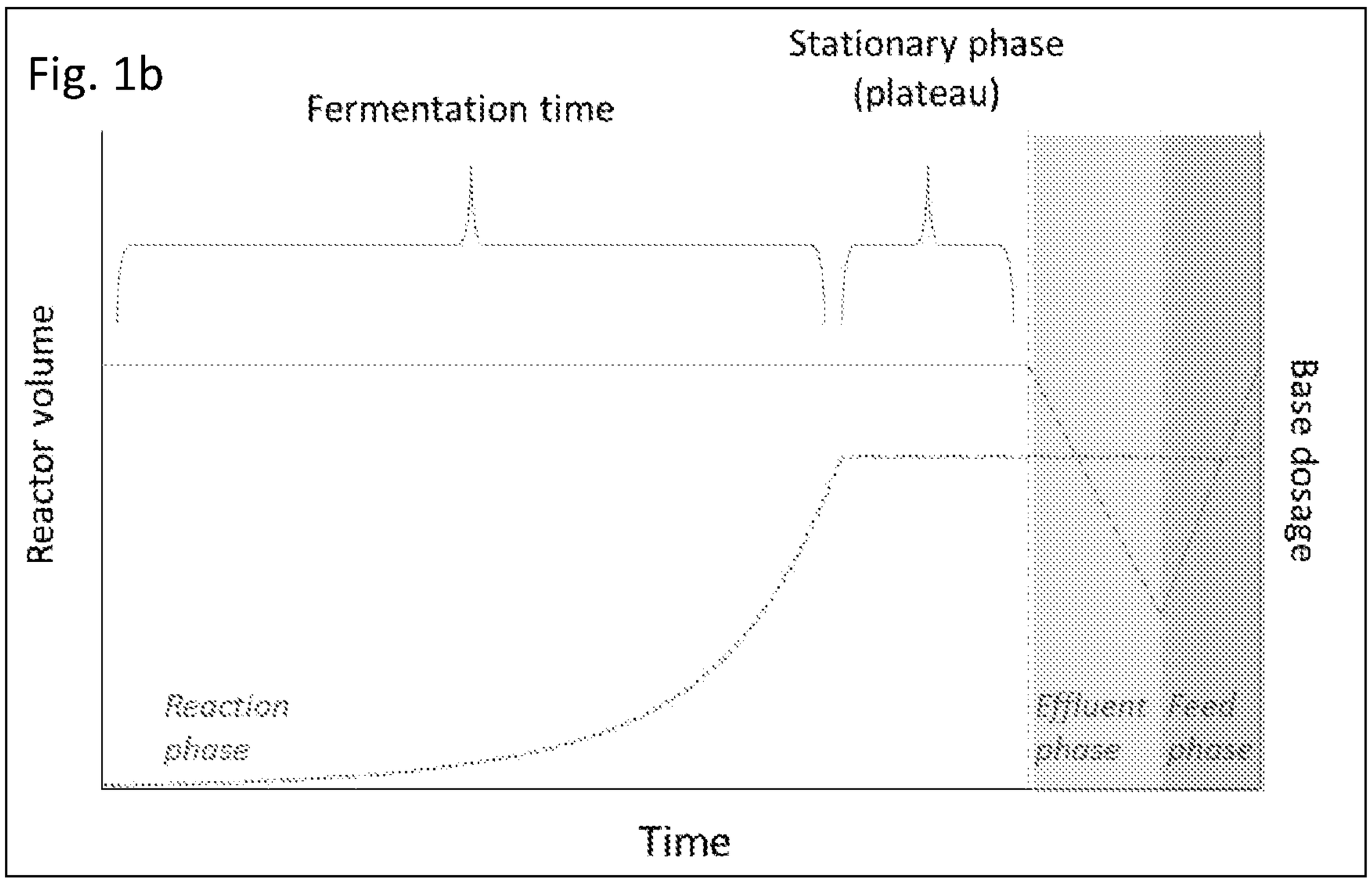

pH
SBR
Reaction
exchange
Effluent
saccharide
Feed

Fig. 1b


Fermentation time
Stationary phase
(plateau)
Reactor volume
Base dosage
Reaction
phase
Effluent
phase
Feed
phase
Time

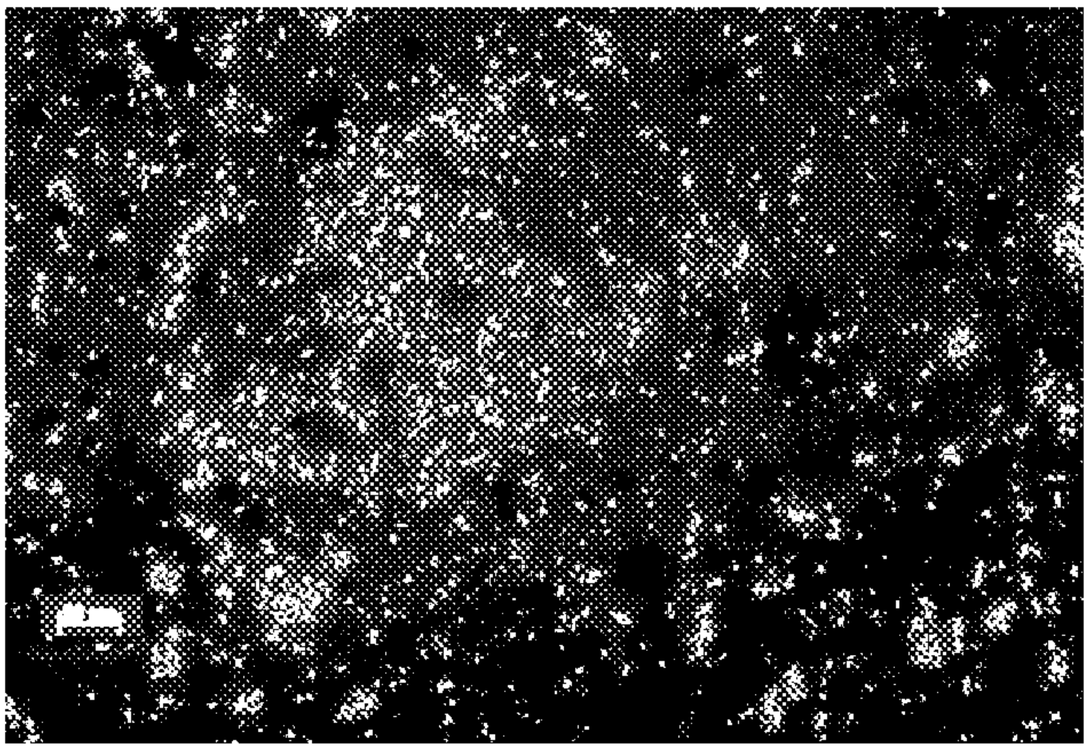


Fig. 2

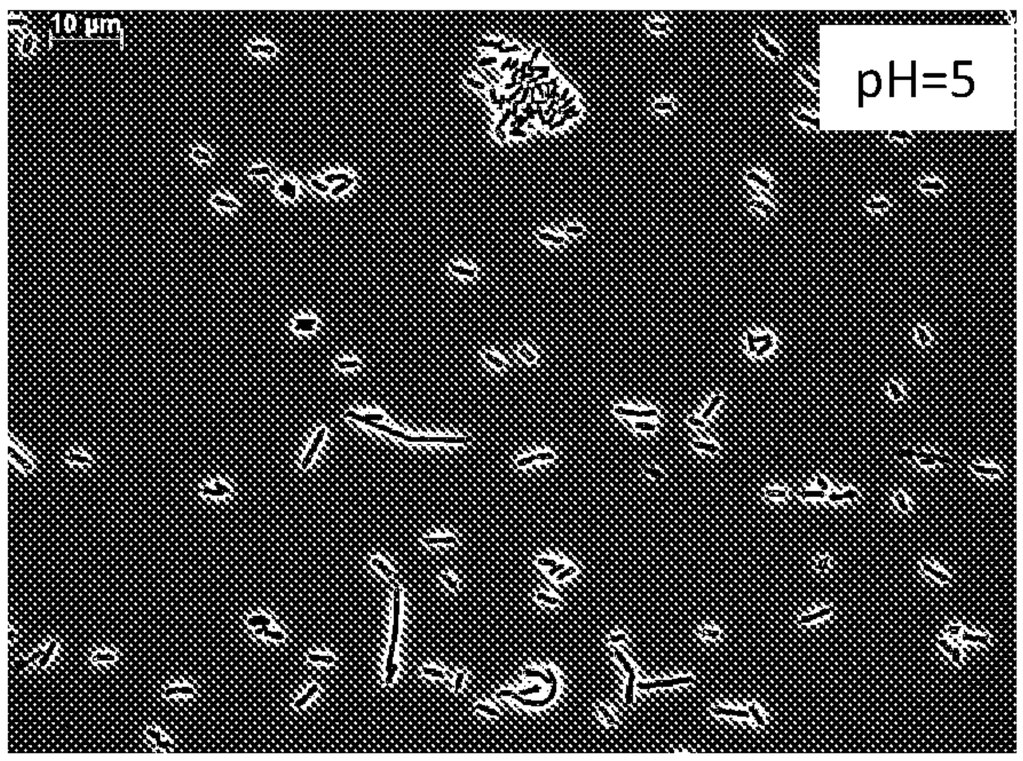


Fig. 3a

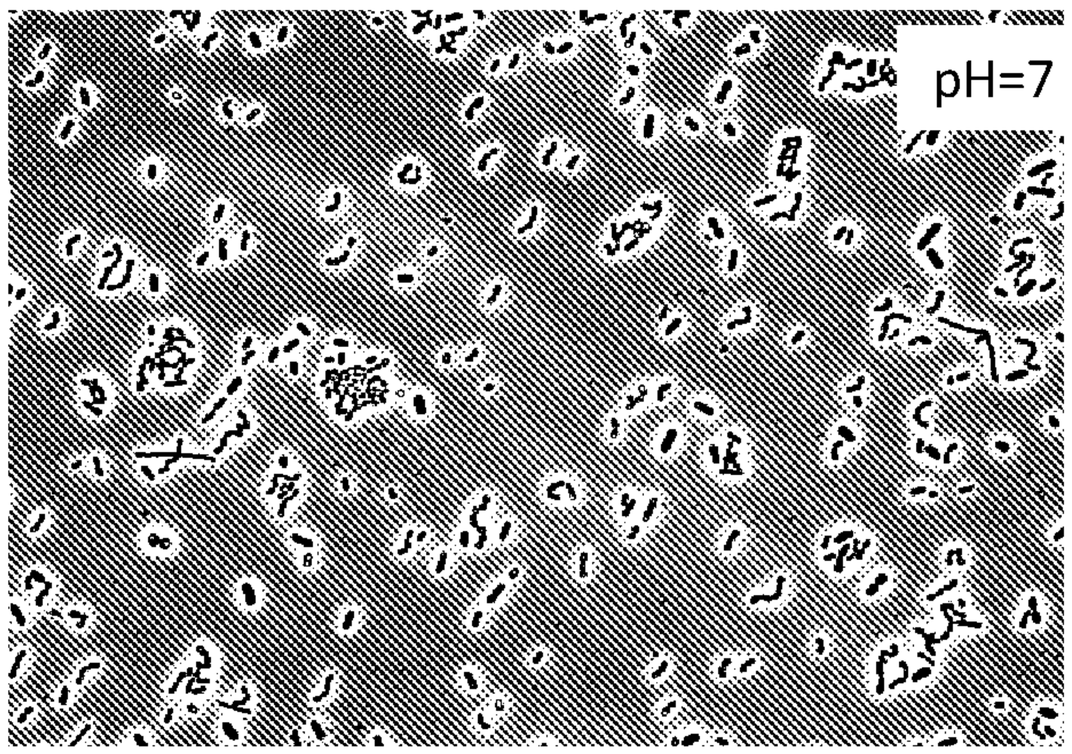


Fig. 3b

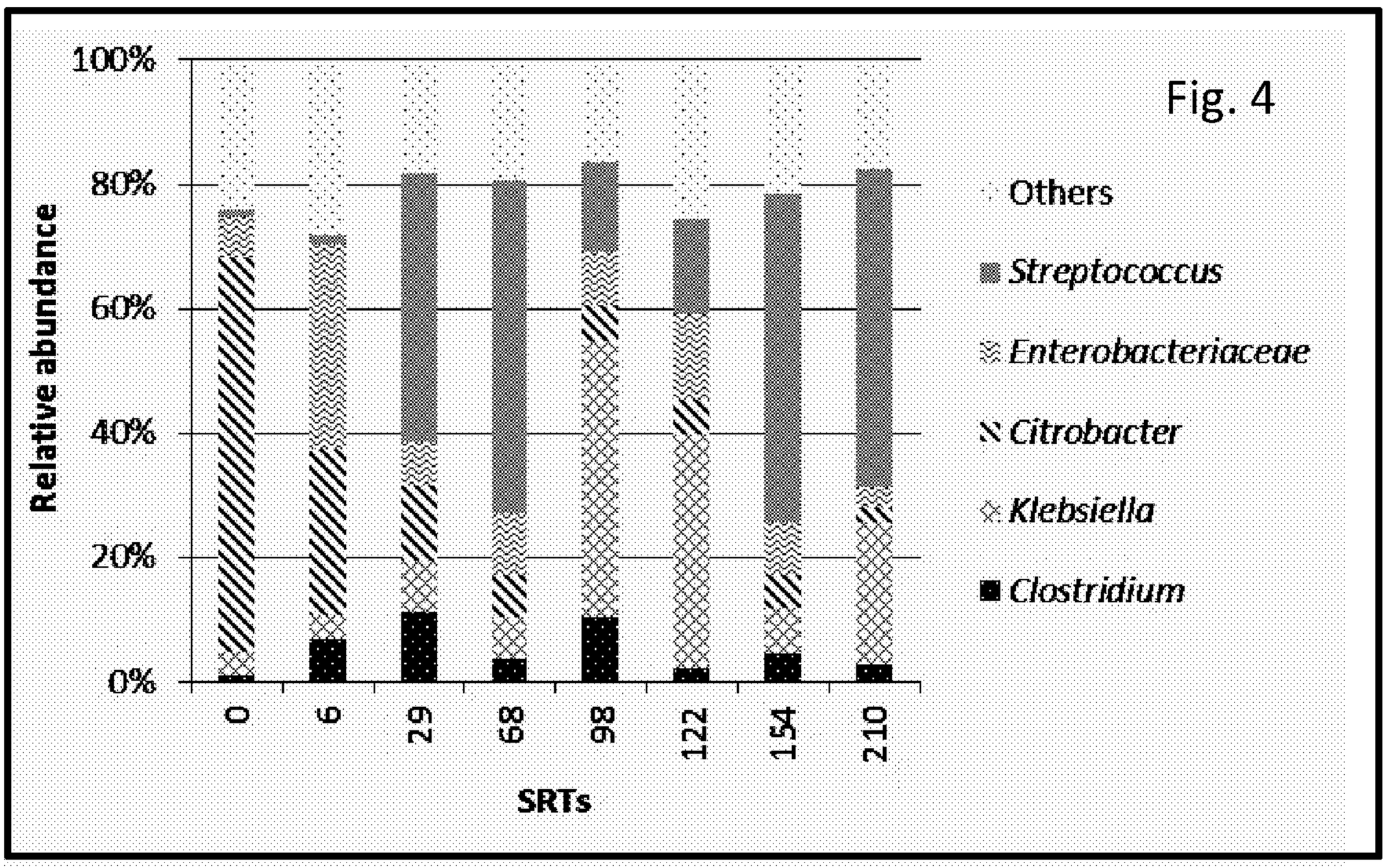


Fig. 4

SAMENWERKINGSVERDRAG (PCT)

RAPPORT BETREFFENDE NIEUWHEIDSONDERZOEK VAN INTERNATIONAAL TYPE

IDENTIFICATIE VAN DE NATIONALE AANVRAGE	KENMERK VAN DE AANVRAGER OF VAN DE GEMACHTIGDE **018541 NL-PD-LV**
Nederlands aanvraag nr. **2023113**	Indieningsdatum **10-05-2019**
	Ingeroepen voorrangsdatum
Aanvrager (Naam) **Technische Universiteit Delft**	
Datum van het verzoek voor een onderzoek van internationaal type **08-06-2019**	Door de Instantie voor Internationaal Onderzoek aan het verzoek voor een onderzoek van internationaal type toegekend nr. **SN73849**

I. CLASSIFICATIE VAN HET ONDERWERP (bij toepassing van verschillende classificaties, alle classificatiesymbolen opgeven)

Volgens de internationale classificatie (IPC)

Zie onderzoeksrapport

II. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte minimumdocumentatie

Classificatiesysteem	Classificatiesymbolen
IPC	**Zie onderzoeksrapport**

Onderzochte andere documentatie dan de minimum documentatie, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

III.	[ ]	**GEEN ONDERZOEK MOGELIJK VOOR BEPAALDE CONCLUSIES**	(opmerkingen op aanvullingsblad)
IV.	[X]	**GEBREK AAN EENHEID VAN UITVINDING**	(opmerkingen op aanvullingsblad)

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2023113

A. CLASSIFICATIE VAN HET ONDERWERP
INV. C12P7/56 C12M1/34
ADD.

Volgens de Internationale Classificatie van octrooien (IPC) of zowel volgens de nationale classificatie als volgens de IPC.

B. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte miminum documentatie (classificatie gevolgd door classificatiesymbolen)
C12M C12P

Onderzochte andere documentatie dan de mimimum documentatie, voor dergelijke documenten, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

Tijdens het onderzoek geraadpleegde elektronische gegevensbestanden (naam van de gegevensbestanden en, waar uitvoerbaar, gebruikte trefwoorden)
EPO-Internal, BIOSIS, COMPENDEX, EMBASE, FSTA, WPI Data

C. VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
	EENHEID VAN UITVINDING ONTBREEKT zie aanvullingsblad B -----	
X	AKAO ET AL: "Semi-continuous l-lactate fermentation of garbage without sterile condition and analysis of the microbial structure", WATER RESEARCH, ELSEVIER, AMSTERDAM, NL, deel 41, nr. 8, 23 maart 2007 (2007-03-23) , bladzijden 1774-1780, XP005935305, ISSN: 0043-1354 * samenvatting * * bladzijde 1775 - bladzijde 1778; figuur 2; tabellen 1-4 * -----	1-19
	-/--	

[X] Verdere documenten worden vermeld in het vervolg van vak C.

[X] Leden van dezelfde octrooifamilie zijn vermeld in een bijlage

° Speciale categorieën van aangehaalde documenten

"A" niet tot de categorie X of Y behorende literatuur die de stand van de techniek beschrijft

"D" in de octrooiaanvrage vermeld

"E" eerdere octrooi(aanvrage), gepubliceerd op of na de indieningsdatum, waarin dezelfde uitvinding wordt beschreven

"L" om andere redenen vermelde literatuur

"O" niet-schriftelijke stand van de techniek

"P" tussen de voorrangsdatum en de indieningsdatum gepubliceerde literatuur

"T" na de indieningsdatum of de voorrangsdatum gepubliceerde literatuur die niet bezwarend is voor de octrooiaanvrage, maar wordt vermeld ter verheldering van de theorie of het principe dat ten grondslag ligt aan de uitvinding

"X" de conclusie wordt als niet nieuw of niet inventief beschouwd ten opzichte van deze literatuur

"Y" de conclusie wordt als niet inventief beschouwd ten opzichte van de combinatie van deze literatuur met andere geciteerde literatuur van dezelfde categorie, waarbij de combinatie voor de vakman voor de hand liggend wordt geacht

"&" lid van dezelfde octrooifamilie of overeenkomstige octrooipublicatie

Datum waarop het onderzoek naar de stand van de techniek van internationaal type werd voltooid	Verzenddatum van het rapport van het onderzoek naar de stand van de techniek van internationaal type
21 januari 2020	
Naam en adres van de instantie European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	De bevoegde ambtenaar Schröder, Gunnar

1

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2023113

C.(Vervolg). VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
X	TANG JIALING ET AL: "Lactic acid fermentation from food waste with indigenous microbiota: Effects of pH, temperature and high OLR", WASTE MANAGEMENT, ELSEVIER, NEW YORK, NY, US, deel 52, 31 maart 2016 (2016-03-31), bladzijden 278-285, XP029539969, ISSN: 0956-053X, DOI: 10.1016/J.WASMAN.2016.03.034 * samenvatting; tabel 1 * * alineas [2.3.], [3.1.], [3.2.], [3.3.]; figuren 4, 6 *	1-19

A	SHAOBO LIANG ET AL: "Lactic acid production from potato peel waste by anaerobic sequencing batch fermentation using undefined mixed culture", WASTE MANAGEMENT, deel 45, 1 november 2015 (2015-11-01), bladzijden 51-56, XP055659705, US ISSN: 0956-053X, DOI: 10.1016/j.wasman.2015.02.004 * samenvatting * * alinea [2.2.]; figuur 2 *	1-19

A	ZHANG B ET AL: "Enhanced isomer purity of lactic acid from the non-sterile fermentation of kitchen wastes", BIORESOURCE TECHNOLOGY, ELSEVIER, AMSTERDAM, NL, deel 99, nr. 4, 1 maart 2008 (2008-03-01), bladzijden 855-862, XP022620614, ISSN: 0960-8524, DOI: 10.1016/J.BIORTECH.2007.01.010 [gevonden op 2008-03-01] * samenvatting; figuren 1, 2 *	1-19

A	US 2011/210001 A1 (XU NANPING [CN] ET AL) 1 september 2011 (2011-09-01) * samenvatting; conclusies 10-18; figuur 1 *	1-19

GEBREK AAN EENHEID VAN UITVINDING

Octrooiaanvrage Nr.:

SN 73849
NL 2023113

AANVULLINGSBLAD B

De Instantie belast met het uitvoeren van het onderzoek naar de stand van de techniek heeft vastgesteld dat deze aanvrage meerdere uitvindingen bevat, te weten:

1. conclusies: 1-19

Method

---

2. conclusie: 20

Apparatus

Het vooronderzoek werd tot het eerste onderwerp beperkt.

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Informatie over leden van dezelfde octrooifamilie

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2023113

In het rapport genoemd octrooigeschrift	Datum van publicatie	Overeenkomend(e) geschrift(en)	Datum van publicatie
US 2011210001 A1	01-09-2011	CN 101392273 A	25-03-2009
		US 2011210001 A1	01-09-2011
		WO 2010051676 A1	14-05-2010

WRITTEN OPINION

File No.	Filing date *(day/month/year)*	Priority date *(day/month/year)*	Application No.
SN73849	10.05.2019		NL2023113
International Patent Classification (IPC) INV. C12P7/56 C12M1/34			
Applicant Technische Universiteit Delft			

This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☒ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the application
- ☒ Box No. VIII Certain observations on the application

	Examiner
	Schröder, Gunnar

Application number

WRITTEN OPINION

NL2023113

Box No. I Basis of this opinion

1. This opinion has been established on the basis of the latest set of claims filed before the start of the search.

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☐ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☐ on paper

   ☐ in electronic form

   c. time of filing/furnishing:

   ☐ contained in the application as filed.

   ☐ filed together with the application in electronic form.

   ☐ furnished subsequently for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Application number

WRITTEN OPINION

NL2023113

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step, or to be industrially applicable have not been examined in respect of

☐ the entire application

☒ claims Nos. 20

because:

☐ the said application, or the said claims Nos. relate to the following subject matter which does not require a search *(specify)*:

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no search report has been established for the whole application or for said claims Nos. 20

☐ a meaningful opinion could not be formed as the sequence listing was either not available, or was not furnished in the international format (WIPO ST25).

☐ a meaningful opinion could not be formed without the tables related to the sequence listings; or such tables were not available in electronic form.

☐ See Supplemental Box for further details.

Box No. IV Lack of unity of invention

1. The requirement of unity of invention is not complied with for the following reasons:

   see separate sheet

2. This report has been established in respect of the following parts of the application:

   ☐ all parts.

   ☒ the parts relating to claims Nos. (see Search Report)

WRITTEN OPINION

Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Yes:	Claims	2-19
	No:	Claims	1
Inventive step	Yes:	Claims	
	No:	Claims	1-19
Industrial applicability	Yes:	Claims	1-19
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VIII Certain observations on the application

see separate sheet

Re Item IV

Lack of unity of invention

1 Unity of invention

It is considered that there are 2 inventions covered by the claims.

1. Claims: 1-19
Method

2. Claim: 20
Apparatus

The reasons, for which the inventions are not so linked as to form a single general inventive concept, are as follows:

1. First, it is noted that the apparatus of claim 20, although indicated to be "for performing the method of claims 1-19), must be interpreted only as being "suitable" therefore. The claim is not limited to apparatusses to be used in the method. Rather, the claim is a claim towards the apparatus as such.

Therefore, the common matter linking together the independent claims 1 and 20 is formulated without specifying the additional features of the method of claim 1 which are not shared by the apparatus as such.

2. The common matter linking together the independent claims 1 and 20 is the following:

A fermentation system with the following method steps or apparatus features adapted therefore,

addition of a feed solution to a stirred fermentation reactor,

control and maintainance of pH withing a predetermined range in the reactor,

collection of an effluent from the reactor, and isolation of a product therefrom.

3. This common matter does not comprise a single general inventive concept, based on same or corresponding special technical features. Any pH controlled continuous or semi-continuous fermentation method for production of a fermentation product share these features, and countless examples could be cited.

The application therefore lacks unity a priori.

In addition, it is noted that claim 1 was found to lack novelty over the prior art, see the documents D1 and D2 cited below.

4. Hence, the 2 separate inventions or groups of inventions mentioned above are not so linked as to form a single general inventive concept.

-

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1 AKAO ET AL, WATER RESEARCH, vol. 41, no. 8 (2007-03-23), pages 1774-1780

D2 TANG JIALING ET AL, WASTE MANAGEMENT, vol. 52 (2016-03-31), pages 278-

D3 Shaobo Liang ET AL, WASTE MANAGEMENT, vol. 45 (2015-11-01), pages 51-56

D4 ZHANG B ET AL, BIORESOURCE TECHNOLOGY, vol. 99, no. 4 (2008-03-01), pa 855-862

D5 US 2011/210001 A1 (XU NANPING [CN] ET AL) 1 september 2011 (2011-09-01)

2 Novelty

2.1 The present application does not meet the criteria of patentability, because the subject-matter of claim 1 is not new.

2.1.1 The document D1discloses:

A method for producing L-lactate by semi-continuous fermentation of garbage without sterile condition (title). The method comprises a semi-continuous fermentation, corresponding to a sequencing batch fermentation (see page 1775, left-hand column, lines 14-20), wherein a part of the cultural broth is drawn out and the feedstock is supplied approximately every 2 days (TEST 1 and TEST 2, see paragraph 2.3.). Hence, this semi-continuous fermentation corresponds to a "reaction phase" in accordance with claim with a duration of about 2 days. It is implicit that fermentation reaches a kind of "stationary phase" in this time.

Further, according to paragraph 2.3., no recirculation of solids in a drawn broth is conducted. Operational conditions of the three experiments, including pH, hydraulic retention time (HRT) and loading rate, are summarized inTable 3. The fermentations are conducted in a continuously stirred reactor at a temperature of 55 °C and with automatic pH control using NaOH in order to maintain the pH at 6 (Run 3-5 in TEST 1 and Run 1 in TEST2, see paragraph 2.1. and table 3). Hence, the features of steps (ia1)-(ic2) of present claim 1 are present in the method of D1.

The broth withdrawn every 2 days, corresponding to an "effluent", is analysed for lactate content, and the method of anaylsis comprises separating i.e. "removing" lactate from the effluent, in accordance with step (iia) of claim 1. It would be evident for the skilled person that in an industrial process, lactate would systematically be separated from the broth by other sepration techniques.

The broth withdrawn every 2 days is replaced by an equal volume of feedstock, which is a diluted slurry of an artificial, non-sterilized garbage whose composition and nutritional characteristics are shown in tables 1 and 2 (see also paragraph 2.2.). Supplementation of this feedstock corresponds to a "feed phase" in accordance with claim 1. As can be seen from table 2 the feedstock (diluted two-fold as indicated in paragraph 2.2.) will contain about 39 g/l carbohydrates (=saccharide) and about 10,6 g/l protein (=peptide), i.e. about 27 g peptide / 100 g saccharide. The characteristics of the feed mixture as indicated in step (iiia) of claim 1 are thus present.

Further, a mixed starting culture capable of fermenting saccharide into lactic acid is added into the reactor in the startup phase, see paragraph 2.3., which indicates that a fermented broth from another semicontinuous thermophilic acidification reactor was used as a seed.

The subject-matter of claim 1 is therefore not new.

2.1.2 The document D2 discloses another example of a method for producing lactic acid in a sequencing batch fermentation of a garbage feedstock. In this method, highest lactic acid yield and productivity is obtained at 37 °C, pH 6 and an organic loading rate (OLR) of 18 g-TS/L d (see abstract). The semi-continuous fermentation comprises a once-a-day feeding and draw-off, wherein 2 L of broth of the total working volume (10 L) of the reactor are exchanged every day (see paragraph 2.3.

The method of D2 appears to comprise all the features of claim 1, which is therefore not new.

2.2 Dependent claims 2-19 do not seem to contain any features which, in combination with the features of any claim to which they refer, meet the requirements of novelty and/or inventive step, see the documents D1 and D2 and the passages cited in the search report.

2.3 The documents D3-D5 represent further relevant prior art.

D3 discloses lactic acid production from potato peel waste by anaerobic sequencing batch fermentation using undefined mixed culture, however without pH control.

D4 discloses non-sterile fermentation of kitchen wastes at pH controlled to 5, 6, 7 or 8 at temperaturs of 35 °C or 45°C. The observed variation of the isomer purity (L-lactic acid) with pH, temperature and fermentation time change is found to be correlated with changes in the microbial community composition.

D5 is a patent application publication disclosing a cleaning process of producing lactic acid, comprising (see claim 10):

- preparing a saccharification liquid through saccharated materials;

- fermenting with nutritive materials and lactic acid bacteria;

- adjusting the pH using liquid alkali;

- filtrating with a porous membrane;

- reintroducing the lactic acid bacteria in the interception liquid into the porous membrane for recycling,

- subjecting the permeate from membrane filtration to nanofiltration;

- subjecting the permeate from nanofiltration to bipolar electrodialysis to prepare lactic acid, and

- concentrating the lactic acid by using vacuum distillation.

Additional features of this process are that:

- the concentrated solution from nanofiltration and the cleaning liquid from fermentation tank and its affiliated equipment are filtrated and sterilized by using ceramic membrane, and then are reintroduced into the fermentation unit for recycling,

- liquid alkali produced at the same time in the electrodialysis step is reintroduced into the fermentation tank for recycling.

Re Item VIII

Certain observations on the application

3 Clarity

3.1 In claim 1, the feature "stationary phase" is vague and ill defined. It is not clear at which exact time point this phase is reached (e.g. a range of % increase within a specified time frame), and also if "stationary" relates to growth or to product formation (or other parameters of the fermentation which are changing over time).

3.2 In claim 1, for the last mentioned feature "adding a mixed starting culture..." it is not indicated where this is added (assumingly at the start of the reaction phase.

3.3 Finally, claim 1 comprises several "preferred" options: preferred pH range in step (ia1), preferred temperature range in step (ia2), preferred stirring speed in step (ia3), preferred optional step (iib), with again preferred ranges, preferred kinds of saccharide compounds in step (iiia). This creates a multitude of possible combinations, to such an extent that the scope of the claim is not anymore clear and concise. Preferred options should be drafted in seprate dependent claims.

3.4 Concerning claim 2, it is not clear to the examiner whether the claim has an additional feature or not. Claim 1 already indicates "cycling at least once" between the 3 phases (the only possible difference being that the word "biomass" is used in claim 2 in this context.

3.5 In claim 5, "biomass" is listed as a preferred kind of peptide. However, biomass is not a peptide (although it comprises peptides), which raises doubts on the actual meaning of "peptide" in the main claim.

3.6 In claims 9 and 11, the features relating to "precursors" are ill-defined, and the claims are therefore not clear. Any compound, such as CO2 or glucose, could be viewed as a precursor of organic molecules synthesized in a cell.

3.7 In claim 12, it is not clear if the indicated "culture titer" relates to cells wet weight or to any other indication.

3.8 In claim 14, it is not clear what "enriched" means, i.e. how are cultures enriched (conditions, kinds of organisms etc.).

3.9 Concerning claims 15 and 17, it is not clear if the retention time indicated in claim 15 refers to the same HRT as claim 17, or if another kind of (non specified) retention time is meant.

3.10 In claim 19, it appears that the % yield (mol/mol) should be based on moles of carbohydrate.