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(54) Title: HIGH EFFICIENCY FERMENTATION PROCESS

(57) Abstract: The present invention is related with a continuous periodic process for producing organic products such as ethanol, butanol, lactic acid, acetic acid and other base chemicals for further derivatizations. For fermentation are used carbohydrates containing materials, at the presence of cultures yeast or bacterial strain. The fermentation is carried out at conditions which provide the minimal cells growth rate of cultures yeast or bacterial strain in bioreactor and it results practically full conversion of carbohydrates.

Description

HIGH EFFICIENCY FERMENTATION PROCESS

Technical Field

[0001] The present invention relates to a technological process for producing fermentation products such as ethanol, butanol, lactic acid, acetic acid and others by novel continuous periodic fermentation. Carbohydrates from several sources (cereals, potatoes, cassava, molasses etc), as the substrates, can reprocessed with a very high productivity speed and practically all (up to 92%) of the carbohydrates is converted into desired products. The process is makes the conversion of carbohydrates close to calculated theoretical maximum.

[0002] The introduction of periodical fermentation (i.e. the adding substrates and removing of products stepwise) method into continuous process, makes cultivated cells more efficient, as the environment is not stable at all. The adding of secondary culture into fermentation system makes the primary culture more viable, as inducing the competition signals.

Background Art

[0003] Most of the existing industries for the production of organic acids, alcohols and other products by fermentation, are mostly based upon periodic batch operation, which usually carried out at low concentration of cultures yeast or bacterial strain (cells of microorganisms) and substrates (substrate inhibition) during 60-80 hour growth cycles.

[0004] When technologically optimal conversion of carbohydrates containing materials is reached, the fermentation is stopped and metabolites are withdrawn from the broth.

[0005] Such processes are characterized: by low productivity (low production rate of metabolites) and delivery of enormous amounts of waste water, which contain cells of microorganisms, by products, substrate residues etc (i.e. stillage).

[0006] The amount of waste water can be a little reduced when fed-batch fermentation is used. Fed-batch fermentation is distinguished from batch fermentation by the addition, during operations, of a certain amount of fresh substrate and by the consequent withdrawal of a proportioned

amount of broth. The fed-batch fermentation is characterized by a little longer overall time cycle that is certainly acceptable for industrial practice where at present for the effective operating procedures the sterilization after every work cycle, for the preventing of contamination are currently used.

[0007] Nevertheless, such process, as well as process of batch fermentation, is characterized by low productivity (low production rate of metabolites), and delivers enormous amounts of waste water, which contain different residual matter.

[0008] One of the key points to increase the rate production of metabolites by fermentation is to maintain the microbial concentration as high as possible. At the same time, one way of reaching a high concentration of biomass in a fermenter and of decreasing of amounts of waste water, which contain cells of microorganisms, is the application of continuous cell-recycle bioreactors, in particular, cell-recycle membrane bioreactors.

[0009] These systems are mostly composed of continuous, well-stirred reactors either with a built-in membrane module or linked together with a separate membrane unit, most often operating on the basis of microfiltration or ultrafiltration.

[0010] However, the basic problem of the given continuous process is the incomplete conversion of carbohydrates. At that, the result of all attempts of increase a conversion of carbohydrates is decrease of productivity of fermentation.

[0011] It is necessary to note, that a water solution of metabolites, which is removed from a fermenter is free from cells of the microorganisms, but contains carbohydrates. The presence of relatively high amount of non-fermented carbohydrates in outlet of a fermenter essentially complicates process of isolation of target products, and also leads to increase in the cost of production.

[0012] Other of the serious problem of the membrane bioreactor is the difficulties connected with the deterioration of filtration rate in a long run of continuous fermentation because of incessant increase of biomass of cells of microorganisms.

[0013] For stabilization of filtration rate Asakura suggests to remove the microbial cake on the filter with periodic back flows of the fermentation gas through the filter elements. (T. Asakura and K. Toda, "New cell recycle ethanol fermentation with periodic cleaning of filter with gas" Bioprocess Engineering 7, 1991, 83-88). However, such actions move the process in the category of periodic processes and at the same time does not allow achieve a high conversion of carbohydrates.

[0014] In the patent application **WO 2009/006909** A (NORDBIOCHEM OÜ) 15.09.2009 , is described the concept of fermentation at minimal cell growth rate with continuous culturing in a cell-recycle continuous fermentor whereby the productivity lactic acid is of 50g/L/h. In comparison with the named before the patent, in present invention there is used periodic growth method, what induces the microbes for the maximal efficiency. Another point is the secondary culture adding into fermentation media, what elevates too the primary culture efficiency. Moreover, this secondary culture induces the viability of the primary culture, thus lowers the costs for the fermentation system sterilization and for the growing of the inoculation material for starting of new production cycle.

Disclosure of Invention

[0015] The purpose of the present invention is carrying out of fermentation process under such conditions at which: high productivity ensured, and practically full conversion of carbohydrates is reached.

[0016] According to present invention, organic products such as ethanol, butanol, lactic acid, acetic acid and other products can be produced in a simple and inexpensive manner by eliminating the disadvantages of the processes discussed above.

[0017] It has been surprisingly found, that if a process is carried out in periodic conditions at sufficiently high concentration of cells and in the conditions which provide very low growth rate of cells of microorganisms in fermenter, it is possible repeatedly to reuse cells of microorganisms isolated from a broth after each periodic stage of a fermentation on the following stage of a fermentation for conversion of a fresh party of carbohydrates.

[0018] It allows to carry out a fermentation with high rate (high productivity) and

with practically full conversion of carbohydrates. This process essentially reduce both, the amount of wastewater and biomass, removed from a fermenter (so-called cell bleed) and, as consequence, to reduce a costs for its utilization.

[0019] It has been revealed, that for each microorganism there are optimal conditions - temperature, pH, amounts of nutrients and bacteriostatics fed into the fermenter, at which growth rate of cells is minimal but sufficient for replacement cells death. At the same time, the growth rate of cells can be reduced by increase of temperature and decrease of pH up to critical and supercritical values for each microorganism and as well by decrease of amounts nutrients fed into the fermenter and by use such of additives as bacteriostatics. Usually, the optimal growth rate of cells reach by combination of stirring and filtration speeds, temperature and pH rates, and by amount of needed amounts of nutrients and bacteriostatics fed into the fermenter.

[0020] It is necessary to note, that existing modern computer-controlled devices of measuring and control allow to automate such a complicated periodic process completely.

Best Mode for Carrying Out the Invention

[0021] According to the present invention each periodic stage of production of organic products by fermentation comprises three steps:

1) loading a water solution of carbohydrates, nutrients, bacteriostatics, salts and cells of microorganisms to fermenter, concentration of cultures yeast or bacterial strain in a fermenter is maintained approximately on fixed state between 5-100 grams dry cell per litre (preferably 20-100 grams dry cell per litre);

2) fermentation with formation metabolites (organic products);

3) removal from fermenter a cell-free water solution metabolites, with re-direction to fermenter the concentrated in filter unit suspension of cells of microorganisms.

[0022] After that in fermenter, process containing with the concentrated suspension of cells (remained after the third stage), load a fresh portion of a water solution of carbohydrates, and if it is necessary, nutrients,

bacteriostatics and salts, then repeat above operations (steps 2 and 3).

[0023] In spite of the fact that ten periodic stages are described in mentioned below examples, however the number of stages can be more than hundred and at that all stages are carried out without additives of fresh cells of microorganisms. This fact is an additional powerful costs saving point, as there is no need for the periodic cultivation of the inoculum (starting culture of fermentation, mostly 10% of fermentation volume).

[0024] It is necessary to note, that when periodic stages are repeated many times, nevertheless the small increase in concentration of cells of microorganisms takes place. Result of it is the increase in viscosity of a broth and reduction of rate of separation of cell from metabolites.

[0025] For elimination of this problem, periodically (after approximately 50-80 periodic stages have been carried out) a small amount of the concentrated suspension of cells of microorganisms is removed from fermenter to create concentration of cells in fermenter of approximately equal to initial concentration of cells (as at the first stage of fermentation).

[0026] The amount of the concentrated suspension of cells removed from a fermenter are essentially less in comparison with a stream of cells at continuous realization a fermentation (so-called “cell bleed”), not to mentioning about conventional periodic process of a batch-fermentation.

[0027] During the removing of the cell suspension, there are most of the non-soluble metabolites; part of cells lysis products etc is too removed from the process.

[0028] For carrying out of the given process a fermenter connected with a cell-recycle device can be used. Preferably, a cell-recycle device is a membrane module.

[0029] The given process allows to produce organic products with high productivity (up to 200 g/L/h) and in this conditions the amount of waste water, which contain cells of microorganisms, does not exceed values of 10 gram per 1 kg of a target product.

[0030] For the preventing of the drastic loss of the fermentation yield after repeated cycles (so called resting), which depends at the microorganism used, there is possibly to add secondary culture to the fermentation broth.

This culture added about in 3/4 process cycles carried out, and the amount added culture is up to 5% of the fermentation volume. During the continuing the fermentation, the main culture is suppressing the added one and the adding of the secondary culture can used again. Described action makes the usage of main culture 5-10 times longer and will have direct economic effect. The principle of phenomena by the data of publications is following - in nature microorganism exists as the mosaic films in the surfaces. They use each other metabolism and decay products like as symbionts. In artificial conditions, monoculture in water soluble stage, there is activated some still unknown mechanisms, preventing the uninhibited growth. The secondary culture has the role of compensation the artificial environment pressures.

[0031] The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

[0032] Examples

[0033] The membrane recycle bioreactor system consists of the fermenter (10 L glass vessel) equipped with pH controller, controller of temperature and agitation. The fermenter was coupled in a semi-closed loop configuration to a membrane module (MW cut-off 100 kDa, F=1.8 $м^2$), to separate cells of microorganism from the growth media and fermentation product.

[0034] Step 1.

At the beginning fermenter is load 5.7 l by mixture of the following composition, g/l:

Carbohydrates 30-200

Nutrients 3-6

Salts of K, Na, Mg and other 10-15

Bacteriostatics (optional) 1-6 (mg/l)

and suspension of cells of microorganisms for creation of concentration of cells in fermenter, equal to 5-100 g/l (counting upon dry-cells).

[0035] Step 2.

The process of fermentation is carried out up to practically full conversion of carbohydrates (>99.9%), automatically controlling temperature (temperature range of 37-55 °C) and pH (pH range of 5-7) in fermenter by

the addition of 6-12% NH_4OH. When conversion of carbohydrates will achieve value of 99.9% the separation process is begins. In said step the minimal cells growth rate of cultures yeast or bacterial strain in bioreactor which is sufficient to provide cells death replacement is controlled by a choice of the certain temperature, pH and amount of nutrients feeding into the fermenter and by using of bacteriostatics.

[0036] Step 3.

The process of separation is carried out by passing under pressure the resulted broth via membrane module, collecting a water solution free of cell products and returning the concentrated suspension of cells to fermenter. Process of separation stop, when in a fermenter there will be approximately 10-15 % of a broth from its initial volume. The remained broth is concentrated suspension of cells of microorganisms.

[0037] The fresh solution of carbohydrates containing if it is necessary, nutrients, bacteriostatics and salts, is added to the remained broth and steps 2 and 3 are repeated.

[0038] Glucose, lactose, fructose, galactose, sucrose can be used as carbohydrates. As nutrients peptone, protein extracts and yeast extract can be used. As salt K_2HPO_4, KH_2PO_4, NaCl, Na acetate, $MgSO_4$, $Fe_2(SO_4)_3$, $ZnSO_4$, $MnSO_4$ and others can be used.

[0039] For the elongation of the fermentation (more filtration steps with one starting culture), there was added secondary culture of bacterium to the fermentation broth. The addition is carried out before a losing of the production efficiency of cultures. The best results were given with Lactococcus strain in combination with a Leuconostoc strain.

[0040] Ammonia used for the neutralization of fermentation environment utilized by the bacterium as the nitrogen source. Thus fewer nutrients needed. This makes the product cheaper and filtrate solution clearer. The last point makes the separation process easier, what means cheaper.

[0041] The fermentation method of the present invention can be applied not only to produce of lactic acid and ethanol, but also to the other organic acids, alcohols and esters, such as acetic acid, formic acid, propionic acid, citric acid, malic acid, maleic acid, malonic acid, fumaric acid, succinic acid, butanol, ethyl acetate and others.

[0042] Conditions and results of the fermentation are presented in tables 1, 2 and 3.

[0043] Table 1. The condition and results of fermentation of carbohydrates to lactic acid

Table 1

№	Microorganism	Carbohydrate (C_{CH}^{1})	Nutrients (C_N^{0})	Bacteriostatic (C_B)	Salts (C_s)	T	pH	$C_C^{1,I}$	$C_C^{1,E}$	$C_C^{10,E}$	τ_F^{1}	τ_F^{10}	C_P	G_P^{1}	G_P^{10}	G_P^{A}
1	Lactococcus lactis 3905	Glucose(50)	Peptone(4)	-	PP(4) SC (10)	39	3.3	7	8	14	65	61	44	28	30	29
2	Lactococcus lactis 3905	Lactose(100)	Yeast Extract(5)	-	PP(2) FS(2) SC(8)	39	5.9	34	35	45	18	16	94	152	162	158
3	Lactobacillus lactis 3123	Glucose(30)	Yeast Extract(4)	-	PP(2) FS(0.1) SC(9)	55	5.7	5.5	6	13	78	72	27	15	16	16
4	Lactobacillus lactis 3123	Lactose(90)	Peptone(4)	Erythro-mycin(2)	PP(4) SC (10)	38	5.8	28	30	42	35	31	86	90	97	95
5	Lactobacillus casei 7657	Sucrose(40)	Yeast Extract(4)	Tetra-cycline(1)	PP(3) SC (9)	43	4.9	8	9	17	71	66	38	23	24	24
6	Lactobacillus casei 7657	Sucrose(80)	Yeast Extract(5)	-	PP(4) SC (10)	49	3.9	30	32	40	22	18	77	110	124	119
7	Lactococcus lactis 3905	Sucrose(30) Glucose(40) Lactose(30)	Yeast Extract(10)	-	PP(2) FS(2) SC(8)	39	5.9	21	23	30	30	27	93	108	116	113
8*	Lactococcus lactis 3905	Glucose(25) Glucose(25) Glucose(25) Glucose(25)	Yeast Extract(10)	-	PP(2) FS(2) SC(8)	39	5.9	11	12	14	61	53	94	63	71	68
9**	Lactococcus lactis 3905 Leuconostoc spp.	Glucose(50)	Yeast Extract(5)	-	PP(2) FS(2) SC(8)	39	5.9									

Volume of fermentation (V_F) – 6 L; Conversion of carbohydrates >99.9%; τ_0 = 3 min; τ_S = 10 min; V_{FT} = 5 L;

* - all necessary quantity of carbohydrates has been broken on four parts and are added to fermenter during all period of fermentation every 15 minutes.

** Fermentation example with the secondary bacterial culture, added in 10% fermenter volume at 85 stage of fermentation.

[0044] Table 2. The condition and results of fermentation of carbohydrates to ethanol

Table 2

№	Microorganism	Carbohydrate (C_{CH}^{1})	Nutrients (C_N^0)	Bacteriostatic (C_B)	Salts (C_s)	T	pH	$C_C^{1,I}$	$C_C^{1,E}$	$C_C^{10,E}$	T_F^{1}	T_F^{10}	C_P	G_P^{1}	G_P^{10}	G_P^{A}
1	Klyveromyces fragilis 2578	Glucose (100)	Peptone (4)	Tetracycline (1)	PP(3) SC(9)	28	6.1	15	18	30	75	67	47	27	29	28
2	Klyveromyces fragilis 2578	Sucrose (200)	Yeast Extract (4)	Erythromycin (1)	PP(4) SC(8)	30	5.8	47	51	76	26	22	94	121	134	130
3	Candida pseudotropicalis	Lactose (60)	Protein Extract (5)	-	PP(3) SC(9)	44	5.9	18	21	45	81	70	28	15	17	16
4	Candida pseudotropicalis	Sucrose (120)	Yeast Extract (4)	-	PP(2) SC(9)	32	3.8	52	56	89	27	23	55	69	76	73

Volume of fermentation (VF) – 6 L; Conversion of carbohydrates >99.9%; T_0 = 3 min; T_s = 10min; V_{FT} = 5 L

[0045] Table 3. Ammonia utilization by bacteria during the lactate fermentation

Table 3

№	Microorganism	Carbohydrate (C_{CH}^{1})	Nutrients (C_N^0)	Salts (C_s)	T	pH	$C_C^{1,I}$	$C_C^{1,E}$	Neutralizator (C_S)	Neutralizator (C_E)	G_P^{A}
1	Lactococcus lactis 3905	Glucose (50)	Yeast Extract (5)	PP(2) FS(2) SC(8)	39	5.9	20	24	Ammonia(4)	Ammonia(3.5)	148
2	Lactococcus lactis 3905	Glucose (50)	Yeast Extract (1)	PP(2) FS(2) SC(8)	39	5.9	20	21	Ammonia(4)	Ammonia(3.2)	134
3	Lactococcus lactis 3905	Glucose (50)	Yeast Extract (5)	PP(2) FS(2) SC(8)	39	5.9	20	22	NaOH(4)	NaOH(3.8)	97

Volume of fermentation (VF) – 6 L; Conversion of carbohydrates >99.9%; T_0 = 3 min; T_s = 10min; V_{FT} = 5 L, carried 10 cycles

[0046] Notes in table 1, 2, 3:

T – the temperature of fermentation, °C;

$C_C^{1,I}$ – the initial concentration of cells of microorganisms in fermenter at the first stage of fermentation, gram/Litre (dry cell);

$C_C^{1,E}$ – the concentration of cells of microorganisms in fermenter after the first stage of fermentation, gram/Litre (dry cell);

$C_C^{10,E}$ – the concentration of cells of microorganisms in fermenter after the tenth stage of fermentation, gram/Litre (dry cell);

C_{CH}^{I} – the initial concentration of carbohydrates in fermenter at all stages of fermentation, gram/Litre;

C_N^0 – the concentration of nutrients in fermenter at the first stage of fermentation, gram/Litre;

C_S – the concentration of salts in fermenter at all stages of fermentation, g/L;

C_B – the concentration of bacteriostatics in fermenter at all stages of fermentation, mg/L;

C_S – the concentration of neutralizator in the inlet, M;

C_E – the concentration of neutralizator after the fermentation cycles (average), M;

τ_0 – the duration of batch charging of substrate, min; (its value approximately constant at all stages of fermentation and is approximately equal to 3 min);

τ_F^1 – the duration of the first stage fermentation, min;

τ_F^{10} – the duration of the tenth stage fermentation, min;

τ_S – the duration of separation of products after fermentation, min. (Its value approximately constant at all stages of fermentation and is equal to 10± 2 min);

C_P – the concentration of product in filtrate, gram/Litre, (its value approximately constant after each stage of fermentation);

V_{FT} – the volume of filtrate at all stages of fermentation, L; (its value approximately constant after each stage of fermentation and is equal to 5 L);

G_P^1 – productivity of product on the first stage fermentation, g/(L*h);

G_P^{10} – productivity of product on the tenth stage fermentation, g/(L*h);

G_P^A – average productivity of product for ten stages of fermentation, g/(L*h);

PP – potassium phosphate;

MS – magnesium sulphate;

SF – ferric sulphate;

SM – salts of manganese;

SC – sodium chloride;

$G_P^i = (V_{FT} * C_P)/ (\tau_0 + \tau_F^i + \tau_S)* V_F$

$G_P^A = Ó(V_{FT} * C_P)/ (\Sigma\tau_0 + \Sigma\tau_F^i + \Sigma\tau_S)* V_F$

Claims

1. A high-efficiency continuous periodic process for production organic products by fermentation of carbohydrates containing materials comprising consecutive steps of:

   a) microbiological fermentation of carbohydrates containing materials at the presence of cultures yeast or bacterial strain at conditions depending at the microbe strain and at the co-cultivation parameters, which provide:

   i) the simultaneous saccharification and fermentation (SSF) process;

   ii) the secondary culture added in to the fermentation process for the longevity of the continuous process;

   iii) the minimal cells growth rate of cultures yeast or bacterial strain in bioreactor but sufficient to provide cells death replacement;

   iv) practically the full conversion of carbohydrates;

   v) simple and fast separation cells of microorganisms from metabolites;

   b) separating of resulting biomass with generating of essentially cell-free aqueous solution of metabolites and of essentially metabolite-free concentrate of cells;

   c) reuse of a concentrate of cells for carrying out of process of a fermentation of the fresh portion of carbohydrates containing materials.
2. The process according to claim 1, wherein the fermentation is carried out in a system membrane bioreactor which comprises a fermenter and a membrane separation module.
3. The process according to claim 1, wherein the minimal and sufficient cells growth rate of cultures yeast or bacterial strain is provided by a choice of the certain temperature, pH, amount of nutrients feeding into the fermenter and by using of bacteriostatics.
4. The process according to claim 3, wherein the temperature is in the range of 37-55 C°.
5. The process according to claim 3, wherein the pH is in the range of 5-7.
6. The process according to claim 1, wherein concentration of cultures yeast or bacterial strain in a fermenter is maintained approximately on fixed state between 5-100 grams dry cell per litre, the concentration is in dependence of producer microbe and co-cultivated microbe strains.

7. The process according to the claim 1, wherein the secondary culture is added about in 3/4 process cycles carried out and the amount of added second culture is up to 5% of the fermentation volume.
8. The process according to claim 1, wherein by needs for neutralization (organic acids production) the chemical used is ammonia because it possesses the nitrate source habit.

INTERNATIONAL SEARCH REPORT

International application No
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A. CLASSIFICATION OF SUBJECT MATTER
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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

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, FSTA, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2009/006909 A1 (NORDBIOCHEM OÜ) 15 January 2009 (2009-01-15) cited in the application * See page 3 (lines 21-23) and pages 4-5 (Examples / compare with the Examples of the Applica- tion) * -----	1-8
A	HERIBAN ET AL: "Nutrition and broth alterations in the lactic acid fermentation", ACTA BIOTECHNOLOGICA, vol. 35, 1993, pages 283-288, XP008018991, * See page 286 (NH4OH for neutralization) * ----- -/--	1-8

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ABULESZ ET AL: "Periodic operation of a continuous culture of baker's yeast", BIOTECHNOLOGY AND BIOENGINEERING, vol. 34, 1989, pages 741-749, XP002656210, * See page 741 (Abstract and Introduction) *	1-8
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