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(54) **Titre : UTILISATION DU GLYCEROL AVEC APPORT LIMITE EN GLUCIDES POUR LA FERMENTATION**
(54) **Title: THE USE OF GLYCEROL WITH LIMITED FEED OF CARBOHYDRATES FOR FERMENTATION**

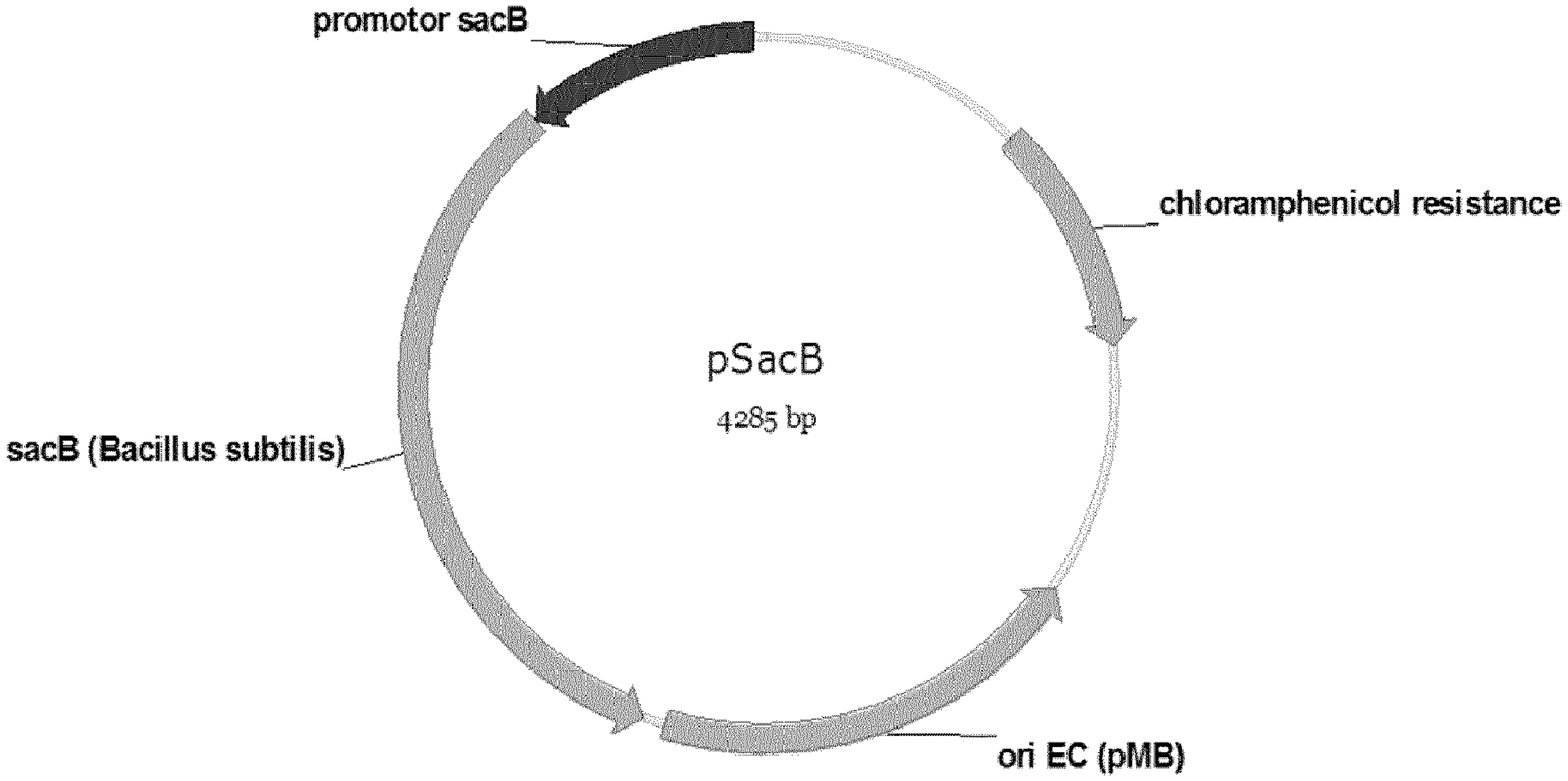

Fig. 1

(57) **Abrégé/Abstract:**

The present invention relates to a process for producing an organic acid by fermentation, comprising the process steps: I)cultivating microorganisms in a culture medium to which are fed, as assimilable carbon sources, glycerol and a further

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carbonaceous compound, to allow the microorganisms to produce the organic acid, thereby obtaining a fermentation broth comprising the organic acid; II) recovering the organic acid or the salt thereof from the fermentation broth obtained in process step I); wherein, at least for a certain period of time in process step I), the consumption rate of the further carbonaceous compound ($C_{Rc.c.}$; in g per liter per hour) is lower than the maximum theoretical consumption rate of the further carbonaceous compound ($CR_{c.c.\ max}$; in g per liter per hour).

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(54) Title: THE USE OF GLYCEROL WITH LIMITED FEED OF CARBOHYDRATES FOR FERMENTATION

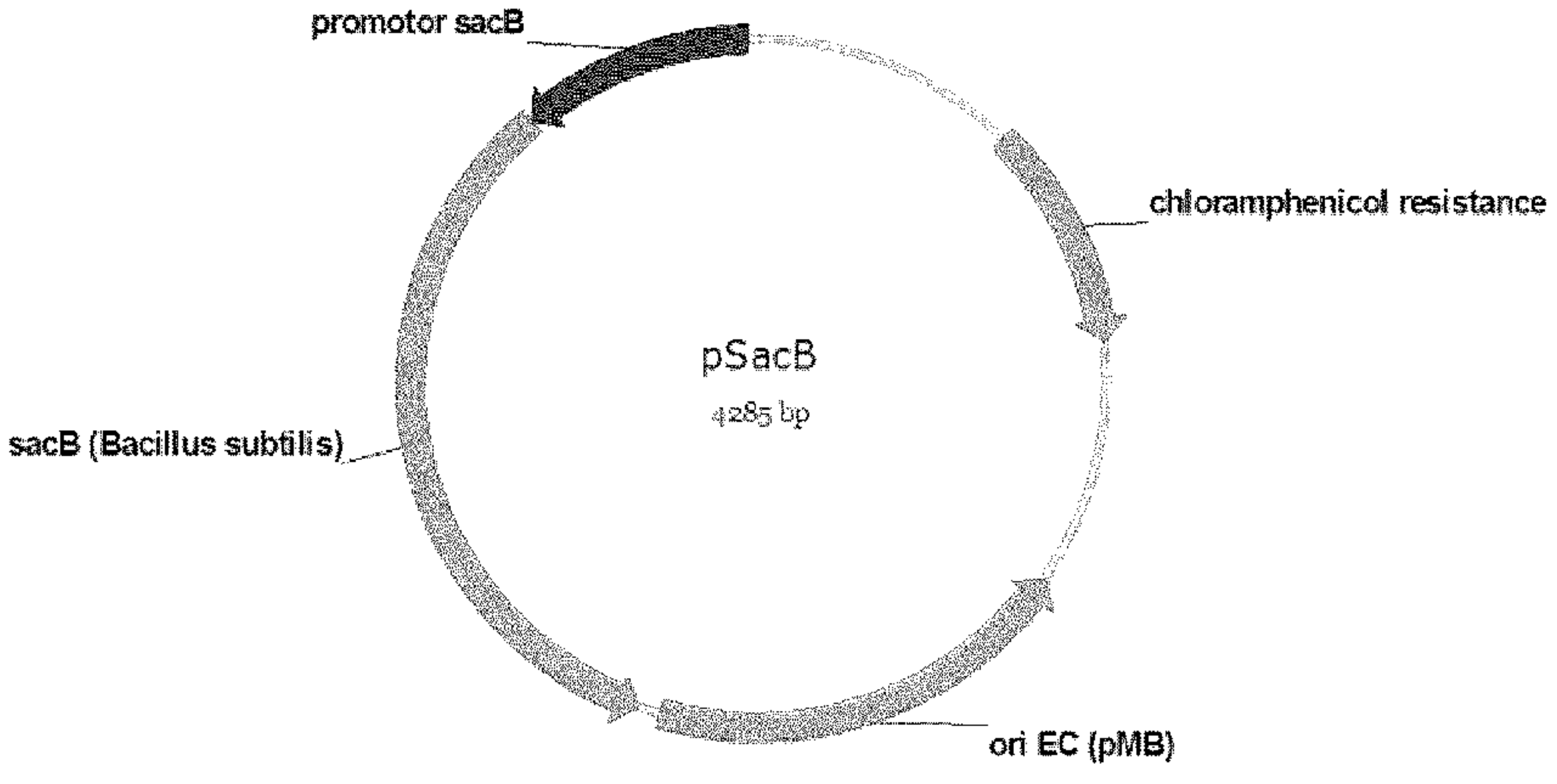

Fig. 1

(57) Abstract: The present invention relates to a process for producing an organic acid by fermentation, comprising the process steps: I)cultivating microorganisms in a culture medium to which are fed, as assimilable carbon sources, glycerol and a further carbonaceous compound, to allow the microorganisms to produce the organic acid, thereby obtaining a fermentation broth comprising the organic acid; II) recovering the organic acid or the salt thereof from the fermentation broth obtained in process step I); wherein, at least for a certain period of time in process step I), the consumption rate of the further carbonaceous compound ($C_{Rc.c.;}$ in g per liter per hour) is lower than the maximum theoretical consumption rate of the further carbonaceous compound ($CR_{c.c.\ max;}$ in g per liter per hour).

- *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description (Rules 13bis.4(d)(i) and 48.2(a)(viii))*
- *with sequence listing part of description (Rule 5.2(a))*

The use of glycerol with limited feed of carbohydrates for fermentation

The present invention relates to a process for producing an organic acid by fermentation.

Organic compounds such as small dicarboxylic acids having 6 or fewer carbons are commercially significant chemicals with many uses. For example, the small diacids include 1,4-diacids, such as succinic acid, malic acid and tartaric acid, and the 5-carbon molecule itaconic acid. Other diacids include the two carbon oxalic acid, three carbon malonic acid, five carbon glutaric acid and the 6 carbon adipic acid and there are many derivatives of such diacids as well.

As a group the small diacids have some chemical similarity and their uses in polymer production can provide specialized properties to the resin. Such versatility enables them to fit into the downstream chemical infrastructure markets easily. For example, the 1,4-diacid molecules fulfill many of the uses of the large scale chemical maleic anhydride in that they are converted to a variety of industrial chemicals (tetrahydrofuran, butyrolactone, 1,4-butanediol, 2-pyrrolidone) and the succinate derivatives succindiamide, succinonitrile, diaminobutane and esters of succinate. Tartaric acid has a number of uses in the food, leather, metal and printing industries. Itaconic acid forms the starting material for production of 3-methylpyrrolidone, methyl-BDO, methyl-THF and others.

In particular, succinic acid or succinate – these terms are used interchangeably herein – has drawn considerable interest because it has been used as a precursor of many industrially important chemicals in the food, chemical and pharmaceutical industries. In fact, a report from the U.S. Department of Energy reports that succinic acid is one of 12 top chemical building blocks manufactured from biomass. Thus, the ability to make diacids in bacteria would be of significant commercial importance.

WO-A-2009/024294 discloses a succinic acid producing bacterial strain, being a member of the family of *Pasteurellaceae*, originally isolated from rumen, and capable of utilizing glycerol as a carbon source and variant and mutant strains derived there from retaining said capability, in particular, a bacterial strain designated DD1 as deposited with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstr. 7B, D-38124 Braunschweig, Germany) having the deposit number DSM 18541 (ID 06-614) and having the ability to produce succinic acid. The DD1-strain belongs to the species *Basfia succiniciproducens* and the family of *Pasteurellaceae* as classified by Kuhnert *et al.*, 2010. Mutations of these strains, in which the *ldhA*-gene and/or the *pflD*- or the *pflA*-gene have been disrupted, are disclosed in WO-A-2010/092155, these mutant strains being characterized by a significantly increased production of succinic acid from carbon sources such as glycerol or mixtures of glycerol and carbohydrates such as maltose, under anaerobic conditions compared to the DD1-wildtype disclosed in WO-A-2009/024294.

However, in the process for producing organic acids as disclosed, for example, in WO-A-2009/024294 or WO-A-2010/092155, the space time yield when using glycerol as the sole carbon source is still improvable.

It was therefore an object of the present invention to overcome the disadvantages of the prior art.

In particular, it was an object of the present invention to provide a process for producing an organic acid, such as succinic acid, by fermentation, which allows the production of these organic acids when using glycerol as the predominant carbon source in higher space time yields, compared to the processes known in the prior art.

A contribution to achieving the abovementioned aims is provided by a process for producing an organic acid by fermentation, comprising the process steps

I) cultivating microorganisms in a culture medium to which are fed, as assimilable carbon sources, glycerol and a further carbonaceous compound, to allow the microorganisms to produce the organic acid, thereby obtaining a fermentation broth comprising the organic acid;

II) recovering the organic acid or the salt thereof from the fermentation broth obtained in process step I);

wherein the consumption rate of the further carbonaceous compound ($CR_{c.c.}$; in g per liter per hour) is lower than the maximum theoretical consumption rate of the further carbonaceous compound ($CR_{c.c.\ max}$; in g per liter per hour).

The inventors of the present application have found that, when producing organic acids such as succinic acid in a fermentation process in which glycerol is used as the predominant carbon source, the space time yield of organic acids such as succinic acid can be significantly improved if a further carbonaceous compound, such as sucrose or D-glucose, is added in a limited way. By adding the further carbonaceous compound in a limited way the consumption rate of the further carbonaceous compound is lower than the maximum theoretical consumption rate of the further carbonaceous compound.

In process step I) of the process according to the present invention microorganisms are cultivated in a culture medium to which are fed, as assimilable carbon sources, glycerol and a further carbonaceous compound, to allow the microorganisms to produce the organic acid, thereby obtaining a fermentation broth comprising the organic acid.

Suitable microorganims according to the present invention may be yeasts, fungi or bacteria. Suitable bacteria, yeasts or fungi are in particular those bacteria, yeasts or fungi which have been deposited at the Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH

(DSMZ), Brunswick, Germany, as bacterial, yeast or fungal strains. Bacteria which are suitable according to the invention belong to the genera detailed under

http://www.dsmz.de/species/bacteria.htm,

yeasts which are suitable according to the invention belong to those genera which are detailed under

http://www.dsmz.de/species/yeasts.htm,

and fungi which are suitable according to the invention are those which are detailed under

http://www.dsmz.de/species/fungi.htm.

Preferably, the microorganisms used in process step I) are bacterial cells. The term "*bacterial cell*" as used herein refers to a prokaryotic organism, i.e. a bacterium. Bacteria can be classified based on their biochemical and microbiological properties as well as their morphology. These classification criteria are well known in the art. According to a preferred embodiment of the process according to the present invention the microorganisms belong to the family of *Enterobacteriaceae*, *Pasteurellaceae*, *Bacillaceae* or *Corynebacteriaceae*.

"*Enterobacteriaceae*" represent a large family of bacteria, including many of the more familiar bacteria, such as *Salmonella* and *Escherichia coli*. They belong to the Proteobacteria, and they are given their own order (Enterobacteriales). Members of the *Enterobacteriaceae* are rod-shaped. Like other Proteobacteria they have Gram-negative stains, and they are facultative anaerobes, fermenting sugars to produce lactic acid and various other end products such as succinic acid. Most also reduce nitrate to nitrite. Unlike most similar bacteria, *Enterobacteriaceae* generally lack cytochrome C oxidase. Most have many flagella used to move about, but a few genera are non-motile. They are non-spare forming, and mostly they are catalase-positive. Many members of this family are a normal part of the gut flora found in the intestines of humans and other animals, while others are found in water or soil, or are parasites on a variety of different animals and plants. *Escherichia coli*, better known as *E. coli*, is one of the most important model organisms, and its genetics and biochemistry have been closely studied. Most members of *Enterobacteriaceae* have peritrichous Type I fimbriae involved in the adhesion of the bacterial cells to their hosts. Examples for the *Enterobacteriaceae* are *E. coli*, *Proteus*, *Salmonella* and *Klebsiella*.

"*Pasteurellaceae*" comprise a large family of Gram-negative Proteobacteria with members ranging from bacteria such as *Haemophilus influenzae* to commensals of the animal and human mucosa. Most members live as commensals on mucosal surfaces of birds and mammals, especially in the upper respiratory tract. *Pasteurellaceae* are typically rod-shaped, and are a notable group of facultative anaerobes. They can be distinguished from the related *Enterobacteriaceae* by the presence of oxidase, and from most other similar bacteria by the absence of flagella.

Bacteria in the family *Pasteurellaceae* have been classified into a number of genera based on metabolic properties and there sequences of the 16S RNA and 23S RNA. Many of the *Pasteurellaceae* contain pyruvate-formate-lyase genes and are capable of anaerobically fermenting carbon sources to organic acids.

"*Bacillaceae*" is a family of Gram-positive, heterotrophic, rod-shaped bacteria that may produce endospores. Motile members of this family are characterized by peritrichous flagellae. Some *Bacillaceae* are aerobic, while others are facultative or strict anaerobes. Most are non-pathogenic, but *Bacillus* species are known to cause disease in humans. This family also comprises the genus *Bacilli* which includes two orders, *Bacillales* and *Lactobacillales*. The bacillus species represents a large cylindrical bacteria that can grow under aerobic conditions at 37°C. They are typically nonpathogenic. The genus *Bacillales* contains the species *Alicyclobacillaceae*, *Bacillaceae*, *Caryophanaceae*, *Listeriaceae*, *Paenibacillaceae*, *Planococcaceae*, *Sporolactobacillaceae*, *Staphylococcaceae*, *Thermoactinomycetaceae*, *Turicibacteraceae*. Many of the *Bacilli* contain pyruvate-formate-lyase genes and are capable of anaerobically fermenting carbon sources to organic acids.

"*Corynebacteriaceae*" is a large family of mostly Gram-positive and aerobic and nonmotile rod-shaped bacteria of the order *Eubacteriales*. This family also comprises the genus *Corynebacterium*, which is a genus of Gram-positive, rod-shaped bacteria. *Corynebacteria* are widely distributed in nature and are mostly innocuous. Some are useful in industrial settings such as *C. glutamicum*.

It is particularly preferred that the microorganism used in process step I) is a modified microorganism. The term "*modified microorganism*" includes a microorganism which has been genetically altered, modified or engineered (e.g., genetically engineered) such that it exhibits an altered, modified or different genotype and/or phenotype (e. g., when the genetic modification affects coding nucleic acid sequences of the microorganism) as compared to the naturally-occurring wildtype microorganism from which it was derived. According to a particular preferred embodiment of the process according to the present invention the modified microorganism used in process step I) is a recombinant microorganism, which means that the microorganism has been obtained using recombinant DNA. The expression "*recombinant DNA*" as used herein refers to DNA sequences that result from the use of laboratory methods (molecular cloning) to bring together genetic material from multiple sources, creating sequences that would not otherwise be found in biological organisms. An example of such a recombinant DNA is a plasmid into which a heterologous DNA-sequence has been inserted.

Preferably, the microorganism, in particular the modified microorganism, used in process step I) has been derived from a wildtype that belongs to the family *Pasteurellaceae*. In this context it is furthermore preferred that the wildtype from which modified microorganism has been derived belongs to the genus *Basfia* and it is particularly preferred that the wildtype from which the modified microorganism has been derived belongs to the species *Basfia succiniciproducens.*

Most preferably, the wildtype from which the modified microorganism used in process step I) has been derived is *Basfia succiniciproducens*-strain DD1 deposited under the Budapest Treaty with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH), Germany, having the deposit number DSM 18541. This strain has been originally isolated from the rumen of a cow of German origin. *Pasteurella* bacteria can be isolated from the gastro-intestinal tract of animals and, preferably, mammals. The bacterial strain DD1, in particular, can be isolated from bovine rumen and is capable of utilizing glycerol (including crude glycerol) as a carbon source. Further strains of the genus *Basfia* that can be used for preparing the modified microorganism according to the present invention are the *Basfia*-strain that has been deposited under the deposit number DSM 22022 with DSZM or the *Basfia*-strains that have been deposited with the Culture Collection of the University of Göteborg (CCUG), Sweden, having the deposit numbers CCUG 57335, CCUG 57762, CCUG 57763, CCUG 57764, CCUG 57765 or CCUG 57766. Said strains have been originally isolated from the rumen of cows of German or Swiss origin.

In this context it is particularly preferred that the wildtype from which the modified microorganism used in process step I) of the process according to the present invention has been derived has a 16S rDNA of **SEQ ID NO: 1** or a sequence, which shows a sequence homology of at least 96 %, at least 97 %, at least 98 %, at least 99 % or at least 99.9 % with **SEQ ID NO: 1**. It is also preferred that the wildtype from which the modified microorganism according to the present invention has been derived has a 23S rDNA of **SEQ ID NO: 2** or a sequence, which shows a sequence homology of at least 96 %, at least 97 %, at least 98 %, at least 99 % or at least 99.9 % with **SEQ ID NO: 2**.

The identity in percentage values referred to in connection with the various polypeptides or polynucleotides to be used for the modified microorganism according to the present invention is, preferably, calculated as identity of the residues over the complete length of the aligned sequences, such as, for example, the identity calculated (for rather similar sequences) with the aid of the program needle from the bioinformatics software package EMBOSS (Version 5.0.0, http://emboss.source-forge.net/what/) with the default parameters which are, i.e. gap open (penalty to open a gap): 10.0, gap extend (penalty to extend a gap): 0.5, and data file (scoring matrix file included in package): EDNAFUL.

It should be noted that the modified microorganism used in process step I) of the process according to the present invention can not only be derived from the above mentioned wildtype-microorganisms, especially from *Basfia succiniciproducens*-strain DD1, but also from variants of these strains. In this context the expression "*a variant of a strain*" comprises every strain having the same or essentially the same characteristics as the wildtype-strain. In this context it is particularly preferred that the 16 S rDNA of the variant has an identity of at least 90 %, preferably at least 95 %, more preferably at least 99 %, more preferably at least 99.5 %, more preferably at least 99.6 %, more preferably at least 99.7 %, more preferably at least 99.8 % and most preferably at least 99.9 % with the wildtype from which the variant has been derived. It is also particularly preferred that the 23 S rDNA of the variant has an identity of at least 90 %, preferably at least 95 %, more preferably at least 99 %, more preferably at least 99.5 %, more preferably at least 99.6 %, more preferably at least 99.7 %, more preferably at least 99.8 % and most preferably at least 99.9 % with the wildtype from which the variant has been derived. A variant of a strain in the sense of this definition can, for example, be obtained by treating the wildtype-strain with a mutagenizing chemical agent, X-rays, or UV light, or by recombinant methods in which genes are deleted, overexpressed or in which heterologous genes are introduced into the cells.

According to a preferred embodiment of the process according to the present invention the modified microorganism used in process step I) is a microorganism that has, compared to its wildtype

i) a reduced pyruvate formate lyase activity,

ii) a reduced lactate dehydrogenase activity, or

iii) a reduced pyruvate formate lyase activity and a reduced lactate dehydrogenase activity.

In context with the expression "*a modified microorganism having, compared to its wildtype, a reduced activity of the enzyme that is encoded by the x-gene*", wherein the *x*-gene is the *ldhA*-gene, the *pflA*-gene and/or the *pflD*-gene, the term "*wildtype*" refers to a microorganism in which the activity of the enzyme that is encoded by the *x*-gene has not been decreased, i. e. to a microorganism whose genome is present in a state as before the introduction of a genetic modification of the *x*-gene (in particular of the *ldhA*-gene, the *pflA*-gene and/or the *pflD*-gene). Preferably, the expression "*wildtype*" refers to a microorganism whose genome, in particular whose *x*-gene, is present in a state as generated naturally as the result of evolution. The term may be used both for the entire microorganism but preferably for individual genes, e.g. the *ldhA*-gene, the *pflA*-gene and/or the *pflD*-gene.

The term "*reduced activity of an enzyme*" as used herein preferably corresponds to a reduction of the activity of the corresponding enzyme by at least 50 %, preferably at least 75 % and more preferably at least 95 %, compared to the activity of the corresponding enzyme in the wildtype.

The term "*reduced activity of an enzyme*" includes, for example, the expression of the enzyme by said genetically modified (e.g., genetically engineered) microorganism at a lower level than that expressed by the wildtype of said microorganism. Genetic manipulations for reducing the expression of an enzyme can include, but are not limited to, deleting the gene or parts thereof encoding for the enzyme, altering or modifying regulatory sequences or sites associated with expression of the gene encoding the enzyme (e.g., by removing strong promoters or repressible promoters), modifying proteins (e.g., regulatory proteins, suppressors, enhancers, transcriptional activators and the like) involved in transcription of the gene encoding the enzyme and/or the translation of the gene product, or any other conventional means of decreasing expression of a particular gene routine in the art (including, but not limited to, the use of antisense nucleic acid molecules or iRNA or other methods to knock-out or block expression of the target protein). Further on, one may introduce destabilizing elements into the mRNA or introduce genetic modifications leading to deterioration of ribosomal binding sites (RBS) of the RNA. It is also possible to change the codon usage of the gene in a way, that the translation efficiency and speed is decreased.

A reduced activity of an enzyme can also be obtained by introducing one or more gene mutations which lead to a reduced activity of the enzyme. Furthermore, a reduction of the activity of an enzyme may also include an inactivation (or the reduced expression) of activating enzymes which are necessary in order to activate the enzyme the activity of which is to be reduced. By the latter approach the enzyme the activity of which is to be reduced is preferably kept in an inactivated state.

Modified microorganisms being deficient in lactate dehydrogenase and/or being deficient in pyruvate formate lyase activity are disclosed in WO-A-2010/092155, US 2010/0159543 and WO-A-2005/052135, the disclosure of which with respect to the different approaches of reducing the activity of lactate dehydrogenase and/or pyruvate formate lyase in a microorganism, preferably in a bacterial cell of the genus *Pasteurella*, particular preferred in *Basfia succiniciproducens* strain DD1, is incorporated herein by reference. Methods for determining the pyruvate formate lyase activity are, for example, disclosed by Asanuma N. and Hino T. in "*Effects of pH and Energy Supply on Activity and Amount of Pyruvate-Formate-Lyase in Streptococcus bovis*", Appl. Environ. Microbiol. (2000), Vol. 66, pages 3773-3777“ and methods for determining the lactate dehydrogenase activity are, for example, disclosed by Bergmeyer, H.U., Bergmeyer J. and Grassl, M. (1983-1986) in "*Methods of Enzymatic Analysis*", 3rd Edition, Volume III, pages 126-133, Verlag Chemie, Weinheim .

In this context it is preferred that the reduction of the activity of lactate dehydrogenase is achieved by an inactivation of the *ldhA*-gene (which encodes the lactate dehydrogenase LdhA; EC 1.1.1.27 or EC 1.1.1.28) and the reduction of the pyruvate formate lyase is achieved by an inactivation of the *pflA*-gene (which encodes for an activator of pyruvate formate lyase PflA; EC 1.97.1.4) or the *pflD*-gene (which encodes the pyruvate formate lyase PflD; EC 2.3.1.54), wherein the inactivation of these genes (i. e. *ldhA*, *pflA* and *pflD*) is preferably achieved by a deletion of theses genes or parts thereof, by a deletion of a regulatory element of these genes or at least a part thereof or by an introduction of at least one mutation into these genes, wherein these modifications are preferably performed by means of the "*Campbell recombination*" as described above.

The *ldhA*-gene the activity of which is reduced in the modified microorganism preferably comprises a nucleic acid selected from the group consisting of:

α1) nucleic acids having the nucleotide sequence of **SEQ ID NO: 3**;

α2) nucleic acids encoding the amino acid sequence of **SEQ ID NO: 4**;

α3) nucleic acids which are at least 70 %, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the nucleic acid of α1) or α2), the identity being the identity over the total length of the nucleic acids of α1) or α2); and

α4) nucleic acids encoding an amino acid sequence which is at least 70 %, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the amino acid sequence encoded by the nucleic acid of α1) or α2), the identity being the identity over the total length of amino acid sequence encoded by the nucleic acids of α1) or α2).

The *pflA*-gene the activity of which is reduced in the modified microorganism preferably comprises a nucleic acid selected from the group consisting of:

β1) nucleic acids having the nucleotide sequence of **SEQ ID NO: 5**;

β2) nucleic acids encoding the amino acid sequence of **SEQ ID NO: 6**;

β3) nucleic acids which are at least 70 %, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the nucleic acid of β1) or β2), the identity being the identity over the total length of the nucleic acids of β1) or β2); and

β4) nucleic acids encoding an amino acid sequence which is at least 70 %, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the amino acid sequence encoded by the nucleic acid of β1) or β2), the identity being the identity over the total length of amino acid sequence encoded by the nucleic acids of β1) or β2).

The *pflD*-gene the activity of which is reduced in the modified microorganism preferably comprises a nucleic acid selected from the group consisting of:

γ1) nucleic acids having the nucleotide sequence of **SEQ ID NO: 7**;

γ2) nucleic acids encoding the amino acid sequence of **SEQ ID NO: 8**;

γ3) nucleic acids which are at least 70 %, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the nucleic acid of γ1) or γ2), the identity being the identity over the total length of the nucleic acids of γ1) or γ2); and

γ4) nucleic acids encoding an amino acid sequence which is at least 70 %, at least 80 %, at least 85 %, at least 90 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 %, at least 99.5 %, at least 99.6 %, at least 99.7 %, at least 99.8 % or at least 99.9 %, most preferably 100 % identical to the amino acid sequence encoded by the nucleic acid of γ1) or γ2), the identity being the identity over the total length of amino acid sequence encoded by the nucleic acids of γ1) or γ2).

In this context it is preferred that the modified microorganism used in process step I) of the process according to the present invention comprises:

A) a deletion of the *ldhA*-gene or at least a part thereof, a deletion of a regulatory element of the *ldhA*-gene or at least a part thereof or an introduction of at least one mutation into the *ldhA*-gene;

B) a deletion of the *pflD*-gene or at least a part thereof, a deletion of a regulatory element of the *pflD*-gene or at least a part thereof or an introduction of at least one mutation into the *pflD*-gene;

C) a deletion of the *pflA*-gene or at least a part thereof, a deletion of a regulatory element of the *pflA*-gene or at least a part thereof or an introduction of at least one mutation into the *pflA*-gene;

D) a deletion of the *ldhA*-gene or at least a part thereof, a deletion of a regulatory element of the *ldhA*-gene or at least a part thereof or an introduction of at least one mutation into the *ldhA*-gene

and

a deletion of the *pflD*-gene or at least a part thereof, a deletion of a regulatory element of the *pflD*-gene or at least a part thereof or an introduction of at least one mutation into the *pflD*-gene;

or

E) a deletion of the *ldhA*-gene or at least a part thereof, a deletion of a regulatory element of the *ldhA*-gene or at least a part thereof or an introduction of at least one mutation into the *ldhA*-gene

and

a deletion of the *pflA*-gene or at least a part thereof, a deletion of a regulatory element of the *pflA*-gene or at least a part thereof or an introduction of at least one mutation into the *pflA*-gene.

Preferably, the organic acid that is produced by the microorganisms in process step I) of the process according to the present invention comprises carboxylic acids such as formic acid, lactic acid, propionic acid, 2-hydroxypropionic acid, 3-hydroxypropionic acid, 3-hydroxybutyric acid, acrylic acid, pyruvic acid or salts of these carboxylic acids, dicarboxylic acids such as malonic acid, succinic acid, malic acid, tartaric acid, glutaric acid, itaconic acid, adipic acid or salts thereof or tricarboxylic acids such as citric acid or salts thereof. According to a particular preferred embodiment of the process according to the present invention the organic acid is succinic acid. The term "*succinic acid*", as used in the context of the present invention, has to be understood in its broadest sense and also encompasses salts thereof (i. e. succinate), as for example alkali metal salts, like Na^+ and K^+-salts, or earth alkali salts, like Mg^{2+} and Ca^{2+}-salts, or ammonium salts or anhydrides of succinic acid.

The microorganisms are preferably incubated in the culture medium at a temperature in the range of about 10 to 60°C or 20 to 50°C or 30 to 45°C at a pH of 2.5 to 9.0 or 3.5 to 8.0 or 4.5 to 7.0.

Preferably, the organic acid, especially succinic acid, is produced under anaerobic conditions. Anaerobic conditions may be established by means of conventional techniques, as for example by degassing the constituents of the reaction medium and maintaining anaerobic conditions by introducing carbon dioxide or nitrogen or mixtures thereof and optionally hydrogen at a flow rate of, for example, 0.1 to 1 or 0.2 to 0.5 vvm. Aerobic conditions may be established by means of conventional techniques, as for example by introducing air or oxygen at a flow rate of, for example, 0.1 to 1 or 0.2 to 0.5 vvm. If appropriate, a slight over pressure of 0.1 to 1.5 bar may be applied in the process.

The process according to the present invention is characterized in that the consumption rate of the further carbonaceous compound ($CR_{c.c.}$; in g per liter per hour) is lower than the maximum theoretical consumption rate of the further carbonaceous compound ($CR_{c.c.\ max}$; in g per liter per hour). The consumption rate of the further carbonaceous compound is lower than the maximum theoretical consumption rate of the further carbonaceous compound if the amount of the further carbonaceous compound (i. e. the amount that is fed into the culture medium and the amount that may already be contained in the culture medium and that is not yet consumed) is lower than the maximum amount of the further carbonaceous compound that the cells could theoretical consume. The amount of the further carbonaceous compound is thus limited.

In the process of the present invention the consumption rate of the further carbonaceous compound can, for example, be controlled by the feeding rate of the further carbonaceous compound (provided that the amount of the further carbonaceous compound that may already be present in the culture medium and that is not yet consumed is sufficiently low). In this context it is preferred that the feeding rate of the further carbonaceous compound is not more than 50 %, more preferably not more than 25 %, more preferably not more than 10 % and most preferably not more than 5 % of the maximum consumption rate of the further carbonaceous compound. Controlling the consumption rate of the further carbonaceous compound by the feeding rate of the further carbonaceous compound is, in particular, possible if the culture medium is essentially free of any (not yet consumed) further carbonaceous compound or if the amount of the further carbonaceous compound is lower than 0.5 g/l, preferably lower than 0.05 g/l, most preferably below the detection limit. In a preferred embodiment the further carbonaceous compound which is fed into the culture medium is immediately and completely consumed by the cells. Under such conditions the further carbonaceous compound is fed in a limited way.

It is furthermore preferred that in the process of the present invention the condition in which the consumption rate of the further carbonaceous compound is lower than the maximum theoretical consumption rate of the further carbonaceous compound is maintained for a certain period of time, preferably for a cultivation period of at least 30 minutes, preferably of at least 1 hour, more preferably of at least 6 hours, even more preferably of at least 12 hours and most preferably of at least 20 hours. In general, the conditions of the limited feed of the further carbonaceous compound are realized for at least 25 %, preferably for at least 50 % and most preferably for at least 70 % of the total cultivation time.

Preferably, the consumption rate of the further carbonaceous compound ($CR_{c.c.}$; in g per liter per hour) is not more than 50 %, more preferably not more than 25 %, more preferably not more than 10 % and most preferably not more than 5 % of the maximum theoretical consumption rate of the further carbonaceous compound ($CR_{c.c.\ max}$; in g per liter per hour). If, for example, the maximum consumption rate for the further carbonaceous compound would be 1 g/L/h, the consumption rate would be not more than 0.5 g/L/h (50 %), preferably not more than 0.25 g/L/h (25 %), more preferably not more than 0.1 g/L/h (10 %) and most preferably not more than 0.05 g/L/h (5 %).

In this context it is furthermore preferred that at the same time (i. e. within the same period of time in which the consumption rate of the further carbonaceous compound is lower than the maximum theoretical consumption rate of the further carbonaceous compound) the amount of glycerol is not limited, but is preferably present in an excess (which means that glycerol is preferably added in such an amount and/or is present in the culture medium in such an amount that the cells cannot consume glycerol faster than it is added to the cell culture and/or than it is present in the culture medium). In this context it is preferred that the consumption rate of glycerol ($CR_{g.}$; in g per liter per hour) is more than 25 %, preferably more than 50 %, more preferably more than 75 %, even more preferably more than 90 % and most preferably more than 95 % of the maximum theoretical consumption rate of glycerol ($CR_{g.\ max}$; in g per liter per hour). If, for example, the maximum consumption rate for glycerol would be 1 g/L/h, the consumption rate would be more than 0.5 g/L/h (50 %), preferably more than 0.75 g/L/h (75 %), more preferably more than 0.9 g/L/h (90 %) and most preferably more than 0.95 g/L/h (95 %).

According to a preferred embodiment of the process according to the present invention, for a cultivation period of at least 30 minutes, preferably of at least 1 hour, more preferably of at least 6 hours, even more preferably of at least 12 hours and most preferably of at least 20 hours

- the consumption rate of the further carbonaceous compound ($CR_{c.c.}$; in g per liter per hour) is not more than 50 %, more preferably not more than 25 %, more preferably not more than 10 % and most preferably not more than 5 % of the maximum theoretical consumption rate of the further carbonaceous compound ($CR_{c.c.\ max}$; in g per liter per hour)

  and, at the same time,

- the consumption rate of glycerol ($CR_{g.}$; in g per liter per hour) is more than 25 %, preferably more than 50 %, more preferably more than 75 %, even more preferably more than 90 % and most preferably more than 95 % of the maximum theoretical consumption rate of glycerol ($CR_{g.\ max}$; in g per liter per hour).

For the determination of the maximum theoretical consumption rate of the further carbonaceous compound $CR_{c.c.\ max}$ and the maximum theoretical consumption rate of glycerol $CR_{g.\ max}$ the cells are incubated in the presence of an excess of the further carbonaceous compound and glycerol, respectively. An excess of the further carbonaceous compound and glycerol is present if, at least for a certain period of time, a certain minimum amount of the not yet consumed substrate (i. e. further carbonaceous compound or glycerol) can be detected in the culture medium, preferably an amount of at least 1 g/L, more preferably at least 2.5 g/L).

For the determination maximum theoretical consumption rate of the further carbonaceous compound $CR_{c.c.\ max}$ the cells are cultured for one hour under exactly the same cultivation conditions as for the determination of $CR_{c.c}$ (i. e. the determintation of the actual consumption rate of the carbonaceous compound when the carbonaceous compound in present in a limited amount), but with an initial amount of carbonaceous compound of at least 5 g per liter and with a further continuous addition of the carbonaceous compound in such an amount that the concentration of the not yet consumed carbonaceous compound in the culture medium will always be above 1 g/L. Furthermore, the expression "*under exactly the same cultivation conditions*" in context with the determination of $CR_{c.c.\ max}$ as used herein indicates that the determination of $CR_{c.c.\ max}$ takes place in the presence of the same amount of glycerol as is present when determining $CR_{c.c}$, but with an excess amount of the carbonaceous compound. For the determination maximum theoretical consumption rate of glycerol $C_{g.\ max}$ the cells are also cultured for one hour under exactly the same cultivation conditions as for the determination of $CR_{g.}$ (i. e. the actual consumption rate of glycerol), but with an initial amount of glycerol as the sole carbon source of at least 5 g per liter and with a further continuous addition of glycerol in such an amount that the concentration of the not yet consumed glycerol in the culture medium will always be above 1 g/L. The expression "*under exactly the same cultivation conditions*" in context with the determination of $CR_{g.\ max}$ as used herein indicates that the determination of $CR_{g.\ max}$ takes place in the presence of the same amount of the further carbonaceous as is present when determining $CR_{g.}$, but with an excess amount of glycerol.

The carbon sources (i. e. glycerol and the further carbonaceous compound) can be added to the cultivation time at once at the beginning of the cultivation or they can be added periodically or continuously. It is, of course, also possible to add, for example, one carbon source (for example glycerol) at once at the beginning of the cultivation and the other carbon source periodically of continuously (for example the further carbonaceous compound). Irrespective the way in which the carbon sources are added to the fermentation medium, it is preferred that the above described conditions for the limited feed of the further carbonaceous compound and the preferably non-limited feed of glycerol are maintained for a cultivation period of at least 30 minutes, preferably of at least 1 hour, more preferably of at least 6 hours, even more preferably of at least 12 hours and most preferably of at least 24 hours.

Preferably, glycerol and the further carbonaceous compound are fed into the culture medium in a total weight ratio glycerol : further carbonaceous compound of at least 5 : 1, more preferably at least 7.5 : 1 and most preferably at least 10 : 1. The term "*total weight ratio*" as used herein defines the ratio of the total amount of the further carbonaceous compound and the total amount of glycerol that are added in process step I).

According to one embodiment of the process according to the present invention the further carbonaceous compound is fed into the fermentation medium in such an amount that the concentration of the further carbonaceous compound in the fermentation medium increases by less than 0.1 g/l/h, preferably less than 0.01 g/l/h. Most preferably, the concentration of the further carbonaceous compound in the fermentation medium does not increase at all (because the added carbonaceous compound is consumed immediately). In this context it is also preferred that concentration of the further carbonaceous compound in the culture medium is between 0 and 1 g/L, preferably less than 0.6 g/L, more preferred less than 0.3 g/L, even more preferred less than 0.1 g/L, wherein most preferably the concentration of the further carbonaceous compound in the culture medium is below the detection limit as measured with a suitable standard assay (preferably by HPLC, as described in the experimental part herein), e.g. determined as a residual concentration in the culture medium, due to the rapid consumption of the further carbonaceous compound by the microorganisms.

The further carbonaceous compound preferably is a carbohydrate except glycerol, more preferably a carbohydrate selected from the groups consisting of sucrose, D-fructose, D-glucose, D-xylose, L- arabinose, D-galactose, D-mannose and mixtures thereof or compositions containing at least one of said compounds. Most preferably, the further carbonaceous compound is selected from the group consisting of sucrose and D-glucose most preferably sucrose, D-glucose and mixtures thereof.

The glycerol used as the assimilable carbon source may by used in the form of pure glycerol or in the form of a crude glycerol that, for example, has been obtained from bio diesel or bio ethanol production without prior purification.

The initial concentration of the assimilable carbon source (i. e. the sum of glycerol and the at least one further carbonaceous compound) is preferably adjusted to a value in a range of 0 to 100 g/l, preferably 0 to 75 g/l, more preferably 0 to 50 g/l, even more preferably 0 to 25 g/l and may be maintained in said range during cultivation (the initial concentration of the assimilable carbon source can be 0 g/l if the further carbonaceous compound is consumed faster than it is fed into the cell culture). The pH of the reaction medium may be controlled by addition of suitable bases as for example, gaseous ammonia, NH_4HCO_3, $(NH_4)_2CO_3$, NaOH, Na_2CO_3, $NaHCO_3$, KOH, K_2CO_3, $KHCO_3$, $Mg(OH)_2$, $MgCO_3$, $Mg(HCO_3)_2$, $Ca(OH)_2$, $CaCO_3$, $Ca(HCO_3)_2$, CaO, $CH_6N_2O_2$, C_2H_7N and/or mixtures thereof. These alkaline neutralization agents are especially required if the organic acids that are formed in the course of the fermentation process are carboxylic acids or dicarboxylic acids. In the case of succinic acid as the organic acid, $Mg(OH)_2$ and $MgCO_3$ are particular preferred bases.

The fermentation step I) according to the present invention can, for example, be performed in stirred fermenters, bubble columns and loop reactors. A comprehensive overview of the possible method types including stirrer types and geometric designs can be found in Chmiel: "*Bioprozesstechnik: Einführung in die Bioverfahrenstechnik*", Volume 1. In the process according to the present invention, typical variants available are the following variants known to those skilled in the art or explained, for example, in Chmiel, Hammes and Bailey: "*Biochemical Engineering*": such as batch, fed-batch, repeated fed-batch or else continuous fermentation with and without recycling of the biomass. Depending on the production strain, sparging with air, oxygen, carbon dioxide, hydrogen, nitrogen or appropriate gas mixtures may be effected in order to achieve good yield (YP/S).

Particularly preferred conditions for producing the organic acid, especially succinic acid, in process strep I) are:

Assimilable carbon source:	glycerol + D-glucose, glycerol + sucrose
Temperature:	30 to 45°C
pH:	5.5 to 8.0
Supplied gas:	CO_2

It is furthermore preferred in process step I) that the assimilable carbon sources (i. e. glycerol and further carbonaceous compound) are converted to the organic acid, preferably to succinic acid, with a carbon yield YP/S of at least 0.75 g/g, preferably of at least 0.85 g/g and most preferably of at least 1.0 g/g, (organic acid/carbon, preferably succinic acid/carbon).

It is furthermore preferred in process step I) that the assimilable carbon sources (i. e. glycerol and further carbonaceous compound) are converted to the organic acid, preferably to succinic acid, with a specific productivity yield of at least 0.6 g g DCW^{-1}h^{-1} organic acid, preferably succinic acid, or of at least of at least 0.65 g g DCW^{-1}h^{-1}, of at least 0.7 g g DCW^{-1}h^{-1}, of at least 0.75 g g DCW^{-1}h^{-1} or of at least 0.77 g g DCW^{-1}h^{-1} organic acid, preferably succinic acid.

It is furthermore preferred in process step I) that the assimilable carbon sources (i. e. glycerol and further carbonaceous compound) are converted to the organic acid, preferably to succinic acid, with a space time yield for the organic acid, preferably for succinic acid, of at least 2.2 g/(L×h) or of at least 2.5 g/(L×h) , at least 2.75 g/(L×h), at least 3 g/(L×h), at least 3.25 g/(L×h), at least 3.5 g/(L×h), at least 3.7 g/(L×h), at least 4.0 g/(L×h) at least 4.5 g/(L×h) or at least 5.0 g/(L×h) of the organic acid, preferably succinic acid. According to another preferred embodiment of the process according to the present invention in process step I) the microorganism is converting at least 20 g/L, more preferably at least 25 g/l and even more preferably at least 30 g/l of the assimilable carbon source (i. e. the sum of glycerol and the at least one further carbonaceous compound) to at least 20 g/l, more preferably to at least 25 g/l and even more preferably at least 30 g/l of the organic acid, preferably succinic acid.

The different yield parameters as described herein ("*carbon yield*" or "*YP/S*"; "*specific productivity yield*"; or "*space-time-yield* (*STY*)") are well known in the art and are determined as described for example by Song and Lee, 2006. "*Carbon yield*" and "*YP/S*" (each expressed in mass of organic acid produced/mass of assimilable carbon source consumed) are herein used as synonyms. The specific productivity yield describes the amount of a product, like succinic acid, that is produced per h and L fermentation broth per g of dry biomass. The amount of dry cell weight stated as "*DCW*" describes the quantity of biologically active microorganism in a biochemical reaction. The value is given as g product per g DCW per h (i.e. g g DCW^{-1}h^{-1}). The space-time-yield (STY) is defined as the ratio of the total amount of organic acid formed in the fermentation process to the volume of the culture, regarded over the entire time of cultivation. The space-time yield is also known as the "*volumetric productivity*".

In process step II) the organic acid, preferably succinic acid, or the salt thereof is recovered from the fermentation broth obtained in process step I).

Usually, the recovery process comprises the step of separating the microorganisms from the fermentation broth as the so called "biomass". Processes for removing the biomass are known to those skilled in the art, and comprise filtration, sedimentation, flotation or combinations thereof. Consequently, the biomass can be removed, for example, with centrifuges, separators, decanters, filters or in a flotation apparatus. For maximum recovery of the product of value, washing of the biomass is often advisable, for example in the form of a diafiltration. The selection of the method is dependent upon the biomass content in the fermentation broth and the properties of the biomass, and also the interaction of the biomass with the organic acid (e. the product of value). In one embodiment, the fermentation broth can be sterilized or pasteurized. In a further embodiment, the fermentation broth is concentrated. Depending on the requirement, this concentration can be done batch wise or continuously. The pressure and temperature range should be selected such that firstly no product damage occurs, and secondly minimal use of apparatus and energy is necessary. The skillful selection of pressure and temperature levels for a multi-stage evaporation in particular enables saving of energy.

The recovery process may further comprise additional purification steps in which the organic acid, preferably succinic acid, is further purified. If, however, the organic acid is converted into a secondary organic product by chemical reactions as described below, a further purification of the organic acid is, depending on the kind of reaction and the reaction conditions, not necessarily required. For the purification of the organic acid obtained in process step II), preferably for the purification of succinic acid, methods known to the person skilled in the art can be used, as for example crystallization, filtration, electrodialysis and chromatography. In the case of succinic acid as the organic acid, for example, succinic acid may be isolated by precipitating it as a calcium succinate product by using calcium hydroxide, -oxide, -carbonate or hydrogen carbonate for neutralization and filtration of the precipitate. The succinic acid is recovered from the precipitated calcium succinate by acidification with sulfuric acid followed by filtration to remove the calcium sulfate (gypsum) which precipitates. The resulting solution may be further purified by means of ion exchange chromatography in order to remove undesired residual ions. Alternatively, if magnesium hydroxide, magnesium carbonate or mixtures thereof have been used to neutralize the fermentation broth, the fermentation broth obtained in process step I)may be acidified to transform the magnesium succinate contained in the medium into the acid form (i. e. succinic acid), which subsequently can be crystallized by cooling down the acidified medium. Examples of further suitable purification processes are disclosed in EP-A-1 005 562, WO-A-2008/010373, WO-A-2011/082378, WO-A-2011/043443, WO-A-2005/030973, WO-A-2011/123268 and WO-A-2011/064151 and EP-A-2 360 137.

According to a preferred embodiment of the process according to the present invention the process further comprises the process step:

I) conversion of the organic acid contained in the fermentation broth obtained in process step I) or conversion of the recovered organic acid obtained in process step II) into a secondary organic product being different from the organic acid by at least one chemical reaction.

In case of succinic acid as the organic acid preferred secondary organic products are selected from the group consisting of succinic acid esters and polymers thereof, tetrahydrofuran (THF), 1,4-butanediol (BDO), gamma-butyrolactone (GBL) and pyrrolidones.

According to a preferred embodiment for the production of THF, BDO and/or GBL this process comprises:

b1) either the direct catalytic hydrogenation of the succinic acid obtained in process steps I) or II) to THF and/or BDO and/or GBL or b2) the chemical esterification of succinic acid and/or succinic acid salts obtained in process steps I) or II) into its corresponding di-lower alkyl ester and subsequent catalytic hydrogenation of said ester to THF and/or BDO and/or GBL.

According to a preferred embodiment for the production of pyrrolidones this process comprises:

b) the chemical conversion of succinic acid ammonium salts obtained in process steps I) or II) to pyrrolidones in a manner known per se.

For details of preparing these compounds reference is made to US-A-2010/0159543 and WO-A-2010/092155.

The invention is now explained in more detail with the aid of figures and non-limiting examples.

Figure 1 shows a schematic map of plasmid pSacB (**SEQ ID NO: 9**).

Figure 2 shows a schematic map of plasmid pSacB Δ*ldhA* (**SEQ ID NO: 10**).

Figure 3 shows a schematic map of plasmid pSacB Δ*pflA* (**SEQ ID NO: 11**).

Figure 4 shows a schematic map of plasmid pSacB Δ*pflD* (**SEQ ID NO: 12**).

EXAMPLES

Example 1: General method for the transformation of *Basfia succiniciproducens*

Strain
Wildtype DD1 (deposit DSM18541)
DD1 Δ*ldhA* Δ*pflA*
DD1 Δ*ldhA* Δ*pflD*

Table 1: Nomenclature of the DD1-wildtype and mutants referred to in the examples

Basfia succiniciproducens DD1 (wildtype) was transformed with DNA by electroporation using the following protocol:

For preparing a pre-culture DD1 was inoculated from frozen stock into 40 ml BHI (brain heart infusion; Becton, Dickinson and Company) in 100 ml shake flask. Incubation was performed over night at 37°C; 200 rpm. For preparing the main-culture 100 ml BHI were placed in a 250 ml shake flask and inoculated to a final OD (600 nm) of 0.2 with the pre-culture. Incubation was performed at 37°C, 200 rpm. The cells were harvested at an OD of approximately 0.5, 0.6 and 0.7, pellet was washed once with 10% cold glycerol at 4°C and re-suspended in 2 ml 10% glycerol (4°C).

100 µl of competent cells were the mixed with 2-8 µg Plasmid-DNA and kept on ice for 2 min in an electroporation cuvette with a width of 0.2 cm. Electroporation under the following conditions: 400 Ω; 25 µF; 2.5 kV (Gene Pulser, Bio-Rad). 1 ml of chilled BHI was added immediately after electroporation and incubation was performed for approximately 2 h at 37°C.

Cells were plated on BHI with 5 mg/L chloramphenicol and incubated for 2-5 d at 37°C until the colonies of the transformants were visible. Clones were isolated and restreaked onto BHI with 5 mg/l chloramphenicol until purity of clones was obtained.

Example 2: Generation of deletion/mutation constructs

Generation of deletions constructs:

Deletion plasmids were constructed based on the vector pSacB (**SEQ ID NO: 9**). Figure 1 shows a schematic map of plasmid pSacB. 5'- and 3'- flanking regions (approx. 1500 bp each) of the chromosomal fragment, which should be deleted were amplified by PCR from chromosomal DNA of *Basfia succiniciproducens* and introduced into said vector using standard techniques. Normally, at least 80 % of the ORF were targeted for a deletion. In such a way, the deletion plasmids for the lactate dehydrogenase *ldhA*, pSacB_delta_*ldhA* (**SEQ ID NO: 10**), the pyruvate formate lyase activating enzyme *pflA*, pSacB_delta_ *pflA* (**SEQ ID NO: 11**) and the pyruvate formate lyase *pflD*, pSacB_delta_*pflD* (**SEQ ID NO: 12**) were constructed. Figures 2, 3 and 4 show schematic maps of plasmid pSacB_delta_*ldhA*, pSacB_delta_*pflA* and pSacB _delta_*pflD*, respectively.

In the plasmid sequence of pSacB (**SEQ ID NO: 9**) the *sacB*-gene is contained from bases 2380-3801. The *sacB*-promotor is contained from bases 3802-4264. The chloramphenicol gene is contained from base 526-984. The origin of replication for *E.coli* (ori EC) is contained from base 1477-2337 (see fig. 1).

In the plasmid sequence of pSacB_delta_*ldhA* (**SEQ ID NO: 10**) the 5' flanking region of the *ldhA* gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 1519-2850, while the 3' flanking region of the *ldhA*-gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 62-1518. The *sacB*-gene is contained from bases 5169-6590. The *sacB*-promoter is contained from bases 6591-7053. The chloramphenicol gene is contained from base 3315-3773. The origin of replication for *E. coli* (ori EC) is contained from base 4266-5126 (see fig. 2).

In the plasmid sequence of pSacB_delta_*pflA* (**SEQ ID NO: 11**) the 5' flanking region of the *pflA*-gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 1506-3005, while the 3' flanking region of the *pflA*-gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 6-1505. The *sacB*-gene is contained from bases 5278-6699. The *sacB*-promoter is contained from bases 6700-7162. The chloramphenicol gene is contained from base 3424-3882. The origin of replication for *E. coli* (ori

EC) is contained from base 4375-5235 (see fig. 3).

In the plasmid sequence of pSacB_delta_*pflD* (**SEQ ID NO: 12**) the 5' flanking region of the *pflD*-gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 1533-2955, while the 3' flanking region of the *pflD*-gene, which is homologous to the genome of *Basfia succiniciproducens*, is contained from bases 62-1532. The *sacB*-gene is contained from bases 5256-6677. The *sacB*-promoter is contained from bases 6678-7140. The chloramphenicol gene is contained from base 3402-3860. The origin of replication for *E. coli* (ori EC) is contained from base 4353-5213 (see fig. 4).

Example 3: Generation of improved succinate producing strains

Generation of deletion mutants:

a) *Basfia succiniciproducens* DD1 was transformed as described above with the pSacB_delta_*ldhA* and "Campbelled in" to yield a "Campbell in" strain. Transformation and integration into the genome of *Basfia succiniciproducens* was confirmed by PCR yielding bands for the integration event of the plasmid into the genome of *Basfia succiniciproducens*.

The "*Campbell in*" strain was then "*Campbelled out*" using agar plates containing sucrose as a counter selection medium, selecting for the loss (of function) of the *sacB*-gene. Therefore, the "*Campbell in*" strains were incubated in 25-35 ml of non selective medium (BHI containing no antibiotic) at 37°C, 220 rpm over night. The overnight culture was then streaked onto freshly prepared BHI containing sucrose plates (10%, no antibiotics) and incubated overnight at 37°C ("first sucrose transfer"). Single colony obtained from first transfer were again streaked onto freshly prepared BHI containing sucrose plates (10%) and incubated overnight at 37°C ("second sucrose transfer"). This procedure was repeated until a minimal completion of five transfers ("third, forth, fifth sucrose transfer") in sucrose. The term "first to fifth sucrose transfer" refers to the transfer of a strain after chromosomal integration of a vector containing a *sacB*-levan-sucrase gene onto sucrose and growth medium containing agar plates for the purpose of selecting for strains with the loss of the *sacB*-gene and the surrounding plasmid sequences. Single colony from the fifth transfer plates were inoculated onto 25-35 ml of non selective medium (BHI containing no antibiotic) and incubated at 37°C, 220 rpm over night. The overnight culture was serially diluted and plated onto BHI plates to obtain isolated single colonies.

The "*Campbelled out*" strains containing either the wildtype situation of the *ldhA*-locus or the mutation/deletion of the *ldhA*-gene were confirmed by chloramphenicol sensitivity. The mutation/deletion mutants among these strains were identified and confirmed by PCR analysis. This led to the *ldhA*-deletion mutant *Basfia succiniciproducens* DD1 *ΔldhA*.

b) *Basfia succiniciproducens* DD1 Δ*ldhA* was transformed with pSacB_delta_*pflA* as described above and "*Campbelled* in" to yield a "Campbell in" strain. Transformation and integration was confirmed by PCR. The "Campbell in" strain was then "*Campbelled out*" as described previously. The deletion mutants among these strains were identified and confirmed by PCR analysis. This led to the *ldhA pflD*-double deletion mutant *Basfia succiniciproducens* DD1 Δ*ldhA* Δ*pflA*.

c) *Basfia succiniciproducens* Δ*ldhA* was transformed with pSacB_delta_*pflD* as described above and "*Campbelled* in" to yield a "*Campbell in*" strain. Transformation and integration was confirmed by PCR. The "*Campbell in*" strain was then "*Campbelled out*" as described previously. The deletion mutants among these strains were identified and confirmed by PCR analysis. This led to the *ldhA pflD*-double deletion mutant *Basfia succiniciproducens* DD1 Δ*ldhA* Δ*pflD*.

Example 4: Determination of glucose or glycerol consumption rate for DD1 Δ*ldhA* Δ*pflA*

1. Medium preparation

The composition of the cultivation medium used for the seed culture is described in table 1. For the main culture fermentation, the medium used is described in table 2.

Compound	Concentration [g/L]
Yeast extract (Bio Springer)	12.5
$(NH_4)_2SO_4$	0.05
succinic acid	2.5
Na_2CO_3	2
KH_2PO_4	1
$MgCO_3$	50
glucose	50

Table 1: Medium composition for cultivation of the seed culture

Compound	Concentration [g/L]
Yeast extract (Bio Springer)	12.5
$(NH_4)_2SO_4$	0.05
betaine	0.23
Na_2CO_3	2
KH_2PO_4	1
Polypropylene glycol (antifoam)	50
glucose	50

Table 2: Medium composition for cultivation of the main culture

2. Cultivations and analytics

The main culture was inoculated after one seed culture step. For the seed culture, the medium described in table 1 was prepared by autoclaving the water, $MgCO_3$ and Na_2CO_3 in a 2L bottle. The other components were prepared and sterilized separated and added to the bottle afterwards in a sterile manner. 1% of cryo stocks were inoculated in a 2L bottle containing 1800 mL of the liquid medium described above. A CO_2 atmosphere was applied in the bottle. The starting pH of the medium was in the range of 7.5 to 8.0 due to the presence of $MgCO_3$ and the CO_2 atmosphere. The incubation was performed at 37°C, 170 rpm (shaking diameter: 2.5 cm) under anaerobic conditions for 12 hours. The culture reached OD_{600nm} of 21.

A total of 5% of the seed culture described above was used to inoculate the main culture. The main culture was performed in 1L-fermenters containing an initial volume of 500 mL of the liquid medium described in table 2. The medium was prepared by autoclaving the water, Na_2CO_3 and antifoam in the fermenter. The other components were prepared separated as solutions and added to the fermenter afterwards in a sterile manner. Glucose was used as the carbon source for these fermentations in order to determine its consumption rate by the succinic acid producing strain. 45 g/L of glucose was batched in the medium and also provided along the fermentation by feeding which was added at a rate of 2 g/L/h. The feeding started 4h after the start of the fermentation. Glucose was in an excess amount during the entire fermentation time and it was measured by HPLC as described in the next section (an excess amount of glucose was confirmed by the fact the that detectable glucose concentration in the fermentation was always above 1 g/L). A pH 6.5 was kept constant during the fermentation and it was controlled by the addition of magnesium hydroxide 15 wt-%. CO_2 was applied in the fermenter at flow of 0.1 vvm and the steering rate was 500 rpm. The analytics of the seed culture and the main culture are described in the next section.

3. Analytics

The production of carboxylic acids was quantified via HPLC. The details about the HPLC method applied are described in table 3 and 4. Cell growth was measured by measuring the absorbance at 600nm (OD_{600nm}) using a spectrophotometer (Ultrospec3000, Amersham Biosciences, Uppsala Sweden).

HPLC column	Aminex HPX-87 H, 300 × 7.8 mm (BioRad)			
Precolumn	Cation H			
Temperature	50 °C			
Eluent flow rate	0.50 ml/min			
Injection volume	5.0 µl			
Diode array detector	RI-Detector			
Runtime	28 min			
max. pressure	140 bar			
Eluent A	5 mM H_2SO_4			
Eluent B	5 mM H_2SO_4			
Gradient	Time [min]	A[%]	B[%]	Flow [ml/min]
	0.0	50	50	0.50
	28.0	50	50	0.50

Table 3: HPLC method (ZX-THF50) for analysis of glycerol, glucose and succinic acid

4. Results

To calculate the glucose consumption rate, fermentation with batched glucose was performed. In this fermentation, glucose was in excess to allow for the calculation of the glucose consumption rate over time. The concentration of glucose was checked offline by HPLC during the fermentation and glucose was always detected in the medium. The measurements confirmed that glucose was always in excess in the medium. In this experiment 45 g/L of glucose was batched and a feeding rate of 2 g/L/h was applied after 4 hours.

Time interval [h]	0 – 9	9 – 15	15 – 23	23 – 40
Glucose consumption rate [g/L/h]	2.76	3.87	2.22	1.61

Table 5: Glucose consumption rate for different time intervals of the succinic acid fermentation with *Basfia* DD1 Δ*ldhA* Δ*pflD* described above

In order to calculate the consumption rate, the amount of glucose consumed in certain time interval was determined.

Example 5: Comparison of fermentations with DD1 Δ*ldhA* Δ*pflA* using glycerol in combination with limited or unlimited glucose

5. Medium preparation

The composition of the cultivation media used for the seed culture and for the main culture are described in the previous section (example 4).

6. Cultivations and analytics

The main culture is inoculated from a seed train consisting of one seed culture. For the seed culture 1% of cryo stocks were inoculated in a 2L bottle with containing 1800 ml of the liquid medium described in table 1 with a CO_2 atmosphere. The starting pH of the medium was in the range of 7.5 to 8.0 due to the presence of $MgCO_3$ and the CO_2 atmosphere. The incubation was overnight at 37°C and 170 rpm (shaking diameter: 2.5 cm) under anaerobic conditions for 12 hours (OD_{600nm} of 21).

A total of 5% of the seed culture was used to inoculate the main culture in 1L-fermenters containing an initial volume of 500 mL of the liquid medium described in table 2. Glycerol and glucose were the carbon sources. 54 g/L of glycerol and 8 g/L of glucose were provided by batch before the start of the fermentation. Glycerol was kept in excess during the entire fermentation and glucose was fed in rates that provided either limited or non-limited amounts of glucose for the cells. The feed is considered limited when the feeding rate is lower than the consumption rate of glucose that was previously determined. The feed started after 4 hours of fermentation and the feeding rate of glucose was either 2.5 g/L/h (unlimited) or 0.25 g/L/h (limited). The base utilized was magnesium hydroxide 15 wt.-%. CO_2 was applied in the fermenter at flow of 0.1 vvm and the steering rate was 500 rpm. The analytics of the seed culture and the main culture have been performed as described in the previous section (table 3). The concentration of glucose was checked offline by HPLC during the fermentation and glucose was always detected in the medium when fed with 2.5 g/L/h. When the limited feed of glucose was applied (0.25 g/L/h), the sugar was not detected in the culture supernatant during a certain time interval.

7. Results

7a. Determination of glucose consumption rate

In this experiment 8 g/L of glucose and 54 g/L of glycerol were batched before the start of the fermentation and a feeding rate of 2.5 g/L/h of glucose were applied after 4 hours of fermentation. The glucose consumption rate was calculated as described in example 4 and the results for each time interval for this specific experimental set up are described in table 6. In this experiment the detectable glucose concentration in the fermentation was always above 1 g/L. The consumption rate for glycose given in table 6 thus represent the "*maximum theoretical consumption rate for the further carbonaceous compound* $CR_{c.c.\ max}$" under the given culture conditions.

Time interval [h]	0 – 9	9 – 15	15 – 23	23 – 40
Glucose consumption rate [g/L/h]	0.94	2.48	2.09	1.29

Table 6: Glucose consumption rate for different time intervals of the succinic acid fermentation with *B. succiniciproducens* DD1 Δ*ldhA* Δ*pflD*

7b. Comparison between limited and unlimited feed of glucose in the succinic acid titer and space time yield of fermentations with *B. succiniciproducens* DD1 Δ*ldhA* Δ*pflD*

After determination of the glucose consumption rate described above, either limited or unlimited feed of glucose were applied in fermentations. The different feeding rates to provide the limited and unlimited glucose concentrations are shown in table 7. The succinic acid titer and space time yield are also shown in table 7.

Feeding rate [g/L/h] [Concentration (g/L)], time (h)	Unlimited Feed 2.5 g/L/h	Limited Feed 0.25 g/L/h
[Glycerol] t=0	54	54
[Glucose] t=0	8	8
[Glucose] t=23h	25	0
[Succinic acid] t=0	1.5	1.5
[Succinic acid] t=23h	25.3	50.6
Space time yield [g/L/h]	1.035	2.135

Table 7: Comparison between limited and unlimited feed of glucose in the production of succinic acid

When feeding glucose with a rate of 0.25 g/L/h (limited feed), the glucose concentration in the fermentation was always below the detection limit.

The results show an increase in the titer of succinic acid produced and a higher space time yield when glucose is added in a limited way.

Example 6: Comparison between different limited feeds of glucose in a fermentation with *B. succiniciproducens* DD1 Δ*ldhA* Δ*pflD*

8. Medium preparation

The compositions of the cultivation media used for the seed culture and for the main culture are described in the section 1 (example 4).

9. Cultivations and analytics

The main culture is inoculated from a seed train consisting of one seed culture. For the seed culture 1% of cryo stocks were inoculated in a 2L bottle with containing 1800 ml of the liquid medium described in table 1 with a CO_2 atmosphere. The starting pH of the medium was in the range of 7.5 to 8.0 due to the presence of $MgCO_3$ and the CO_2 atmosphere. The incubation was overnight at 37°C and 170 rpm (shaking diameter: 2.5 cm) under anaerobic conditions for 12 hours (OD_{600nm} of 21).

A total of 5% of the seed culture was used to inoculate the main culture in 1L-fermenters containing an initial volume of 500 mL of the liquid medium described in table 2. Glycerol and glucose were the carbon sources. 50 g/L of glycerol and 8 g/L of glucose were provided by batch before the start of the fermentation. Glycerol was kept in excess during the entire fermentation and glucose was fed in rates that provided limited amounts of glucose for the cells. The feed is considered limited when the feeding rate is lower than the consumption rate of glucose that was previously determined. The feed started after 4 hours of fermentation and the feeding rates of glucose are described in table 8. The base utilized was magnesium hydroxide 15 wt-%. CO_2 was applied in the fermenter at flow of 0.1 vvm and the steering rate was 500 rpm. The analytics of the seed culture and the main culture have been performed as described in the previous section (table 3). The concentration of glucose was checked offline by HPLC during the fermentation and glucose not detected in the culture supernatant during a certain time interval.

10. Results

After calculating the glucose consumption rate as described in example 1, fermentations with feeding rates of glucose lower than the consumption rate (limited feed) were performed. Glycerol is in excess in the fermentation. The succinic acid titer and the space time yield are directly influenced by the amount of glucose fed into the fermenter (table 8). The results observed in table 8 confirm that in fermentations where glycerol is the main carbon source, the amount of succinic acid and the space time yield are improved with limited amounts of glucose. The lower the feeding rate of glucose, the higher is the succinic acid titer and the space time yield.

Feed rate glucose [g/L/h]	Succinic acid titer [%]	Space time yield [g succinic acid/L/time (h)]
0.93	100	2.5
0.56	105	2.62
0.37	112	2.76

Table 8: Influence of limited feeding rates of glucose in combination with an excess of glycerol on the titer and the space time yield of succinic acid fermentation by *Basfia succiniciproducens* DD1 Δ*ldhA* Δ*pflA*.

The examples show that limited amounts of glucose are beneficial when using glycerol as the main C-source for succinic acid fermentations.

SEQUENCES

SEQ ID NO: 1 (nucleotide sequence of 16 S rDNA of strain DD1)

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PCT

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(This sheet is not part of and does not count as a sheet of the international application)

0-1	**Form PCT/RO/134 Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)**	
0-1-1	Prepared Using	PCT Online Filing Version 3.5.000.241e MT/FOP 20141031/0.20.5.20
0-2	**International Application No.**	
0-3	**Applicant's or agent's file reference**	B75743PC

1	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
1-1	**page**	1
1-2	**line**	31-33
1-3	**Identification of deposit**	
1-3-1	Name of depositary institution	DSMZ Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)
1-3-2	Address of depositary institution	Inhoffenstr. 7B, 38124 Braunschweig, Germany
1-3-3	Date of deposit	11 August 2006 (11.08.2006)
1-3-4	Accession Number	DSMZ 18541
1-4	**Additional Indications**	With respect to the designation of the EPO the applicant hereby declares under Rule 32(1) EPC that the biological material is to be made available only by the issue
1-5	**Designated States for Which Indications are Made**	

FOR RECEIVING OFFICE USE ONLY

0-4	**This form was received with the international application:** (yes or no)	yes
0-4-1	Authorized officer	Kuiper-Cristina, Nathalie

FOR INTERNATIONAL BUREAU USE ONLY

0-5	**This form was received by the international Bureau on:**	
0-5-1	Authorized officer	

CLAIMS

1. A process for producing an organic acid by fermentation, comprising the process steps:
   I) cultivating microorganisms in a culture medium to which are fed, as assimilable carbon sources, glycerol and a further carbonaceous compound, to allow the microorganisms to produce the organic acid, thereby obtaining a fermentation broth comprising the organic acid;
   II) recovering the organic acid or the salt thereof from the fermentation broth obtained in process step I);

   wherein the consumption rate of the further carbonaceous compound ($CR_{c.c.}$; in g per liter per hour) is lower than the maximum theoretical consumption rate of the further carbonaceous compound ($CR_{c.c.\ max}$; in g per liter per hour).

2. Process according to claim 1, wherein for a cultivation period of at least 30 minutes the consumption rate of the further carbonaceous compound ($CR_{c.c.}$; in g per liter per hour) is lower than the maximum theoretical consumption rate of the further carbonaceous compound ($CR_{c.c.\ max}$; in g per liter per hour).

3. Process according to claim 1 or 2, wherein the consumption rate of the further carbonaceous compound ($CR_{c.c.}$; in g per liter per hour) is not more than 50 % of the maximum theoretical consumption rate of the further carbonaceous compound ($CR_{c.c.\ max}$; in g per liter per hour).

4. Process according to anyone of the preceding claims, wherein in process step I) glycerol and the further carbonaceous compound are fed into the culture medium in a total weight ratio glycerol : further carbonaceous compound of at least 5 : 1.

5. Process according to anyone of the preceding claims, wherein the organic acid is succinic acid.

6. Process according to anyone of the preceding claims, wherein the further carbonaceous compound is a carbohydrate.

7. Process according to claim 6, wherein the carbohydrate is selected from the group consisting of sucrose, D-glucose or mixtures thereof.

8. Process according to anyone of the preceding claims, wherein the microorganism used in process step I) is a modified microorganism.

9. Process according to claim 8, wherein the wildtype from which the modified microorganism has been derived belongs to the family of *Pasteurellaceae*.

10. Process according to claim 9, wherein the wildtype from which the modified microorganism has been derived belongs to the genus *Basfia*.

11. Process according to claim 10, wherein the microorganism used in process step I) belongs to the species *Basfia succiniciproducens*.

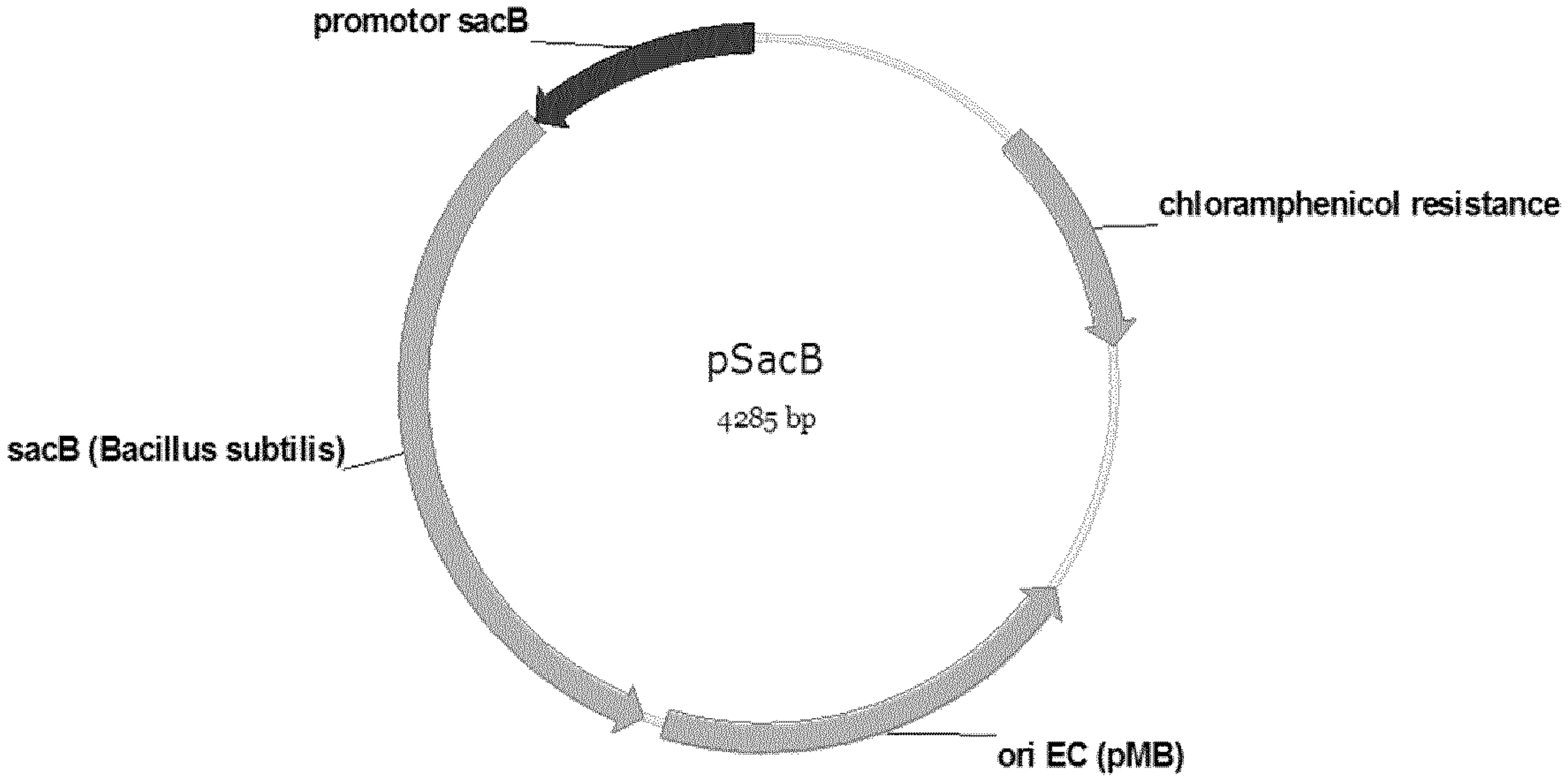
12. Process according to claim 11, wherein the wildtype from which the modified microorganism has been derived has a 16S rDNA of **SEQ ID NO: 1** or a sequence, which shows a sequence homology of at least 96 % with **SEQ ID NO: 1**.

13. Process according to anyone of claims 8 to 12, wherein the modified microorganism has, compared to its wildtype,
    i) a reduced pyruvate formate lyase activity,
    ii) a reduced lactate dehydrogenase activity, or
    iii) a reduced pyruvate formate lyase activity and a reduced lactate dehydrogenase activity.

14. Modified microorganism according to claim 13, wherein the microorganism comprises:
    A) a deletion of the *ldhA*-gene or at least a part thereof, a deletion of a regulatory element of the *ldhA*-gene or at least a part thereof or an introduction of at least one mutation into the *ldhA*–gene;
    B) a deletion of the *pflD*-gene or at least a part thereof, a deletion of a regulatory element of the *pflD*-gene or at least a part thereof or an introduction of at least one mutation into the *pflD*–gene;
    C) a deletion of the *pflA*-gene or at least a part thereof, a deletion of a regulatory element of the *pflA*-gene or at least a part thereof or an introduction of at least one mutation into the *pflA*–gene;
    D) a deletion of the *ldhA*-gene or at least a part thereof, a deletion of a regulatory element of the *ldhA*-gene or at least a part thereof or an introduction of at least one mutation into the *ldhA*–gene
    and
    a deletion of the *pflD*-gene or at least a part thereof, a deletion of a regulatory element of the *pflD*-gene or at least a part thereof or an introduction of at least one mutation into the *pflD*–gene;
    or
    E) a deletion of the *ldhA*-gene or at least a part thereof, a deletion of a regulatory element of the *ldhA*-gene or at least a part thereof or an introduction of at least one mutation into the *ldhA*–gene
    and
    a deletion of the *pflA*-gene or at least a part thereof, a deletion of a regulatory element of the *pflA*-gene or at least a part thereof or an introduction of at least one mutation into the *pflA*–gene.

15. Method according to anyone of preceeding claims, wherein the process further comprises the process step:
    III) conversion of the organic acid contained in the fermentation broth obtained in process step I) or conversion of the recovered organic acid obtained in process step II) into a secondary organic product being different from the organic acid by at least one chemical reaction.

16. Method according to claim 15, wherein the organic acid is succinic acid and wherein the secondary organic product is selected from the group consisting of succinic acid esters or polymers thereof, tetrahydrofuran (THF), 1,4-butanediol (BDO), gamma-butyrolactone (GBL) and pyrrolidones.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chloramphenicol resistance
pSacB
4285 bp
sacB (Bacillus subtilis)
ori EC (pMB)

Fig. 1

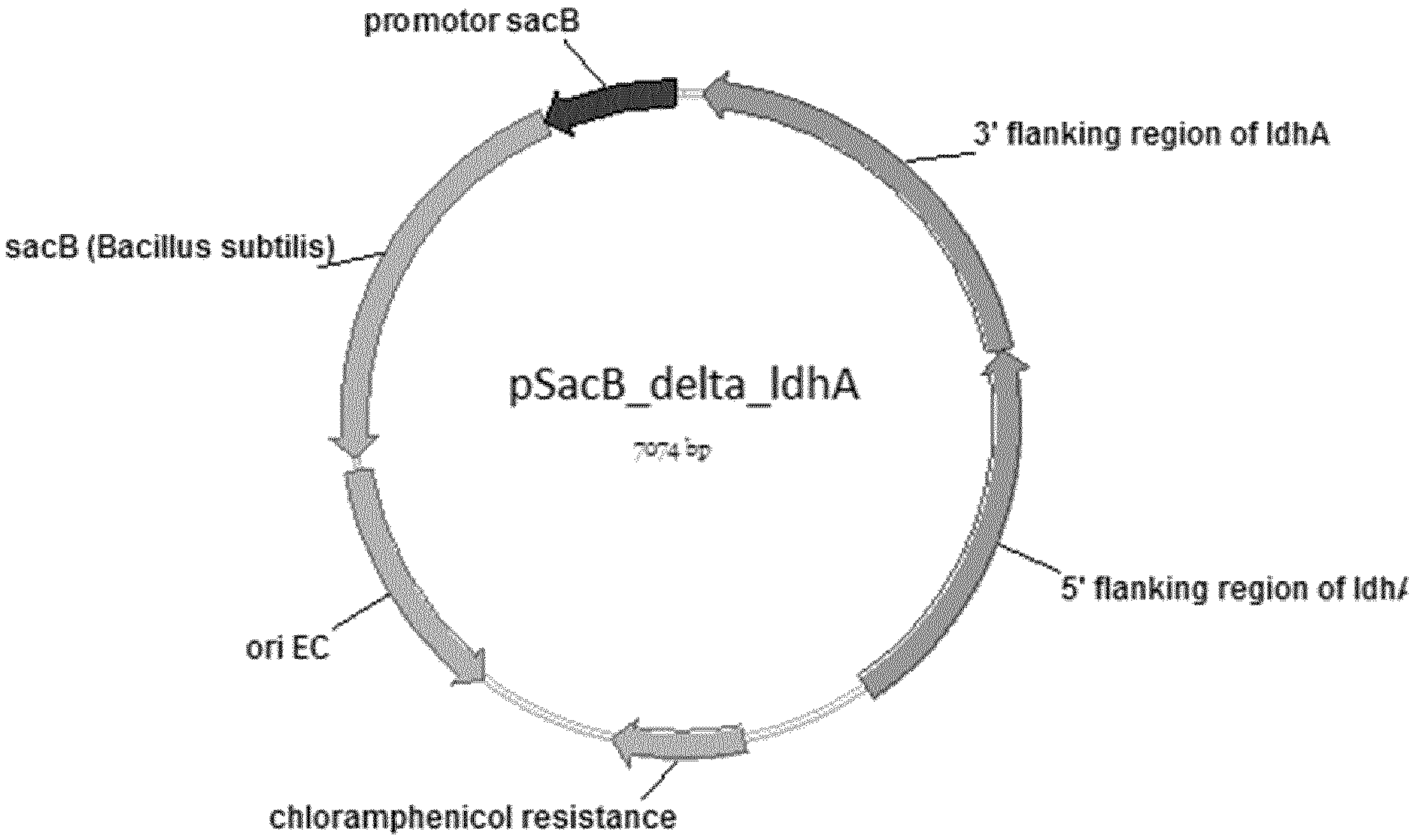
promotor sacB
3' flanking region of ldhA
sacB (Bacillus subtilis)
pSacB_delta_ldhA
7074 bp
5' flanking region of ldh
ori EC
chloramphenicol resistance

Fig. 2

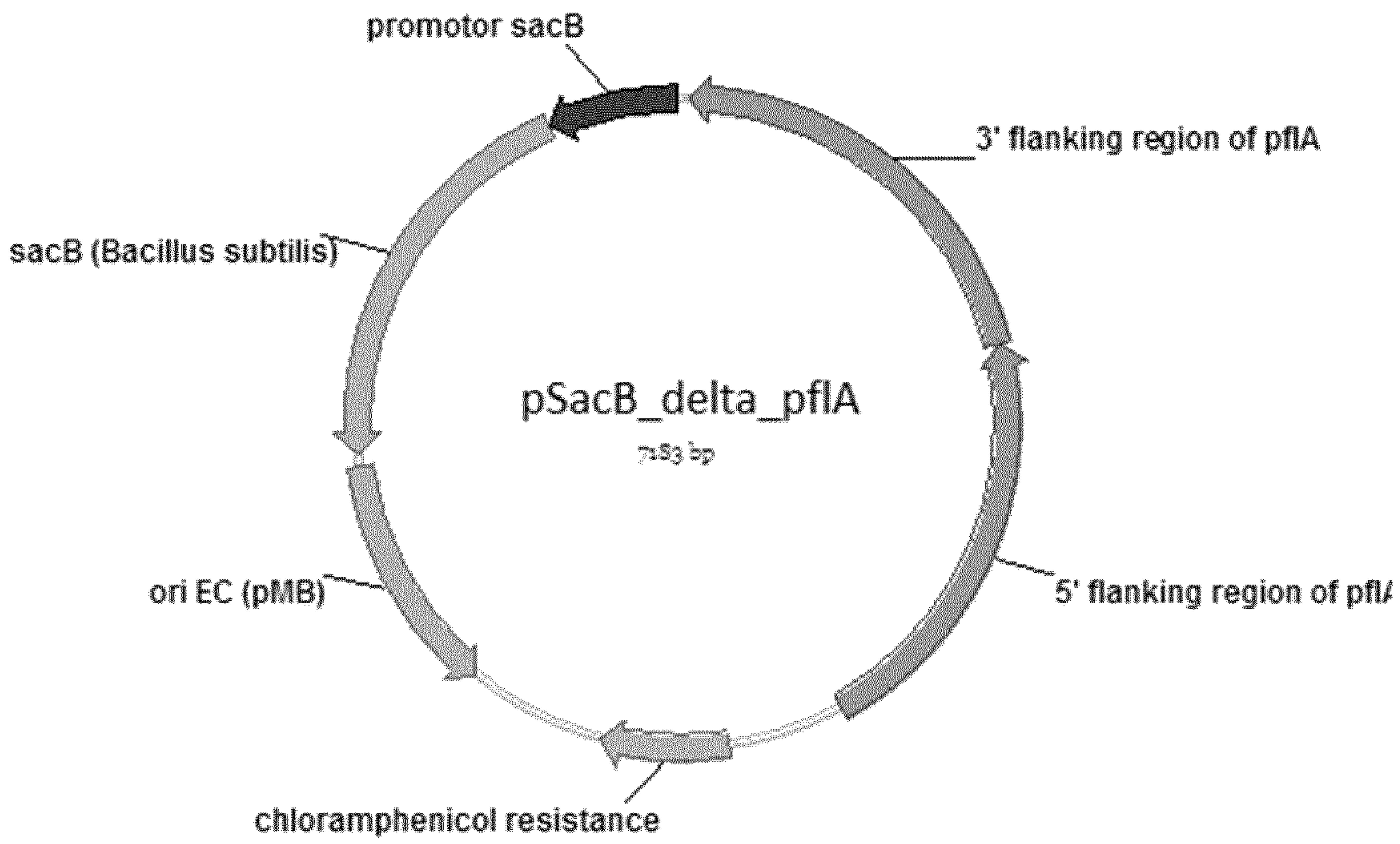
promotor sacB
3' flanking region of pflA
sacB (Bacillus subtilis)
pSacB_delta_pflA
7183 bp
ori EC (pMB)
5' flanking region of pfl
chloramphenicol resistance

Fig. 3

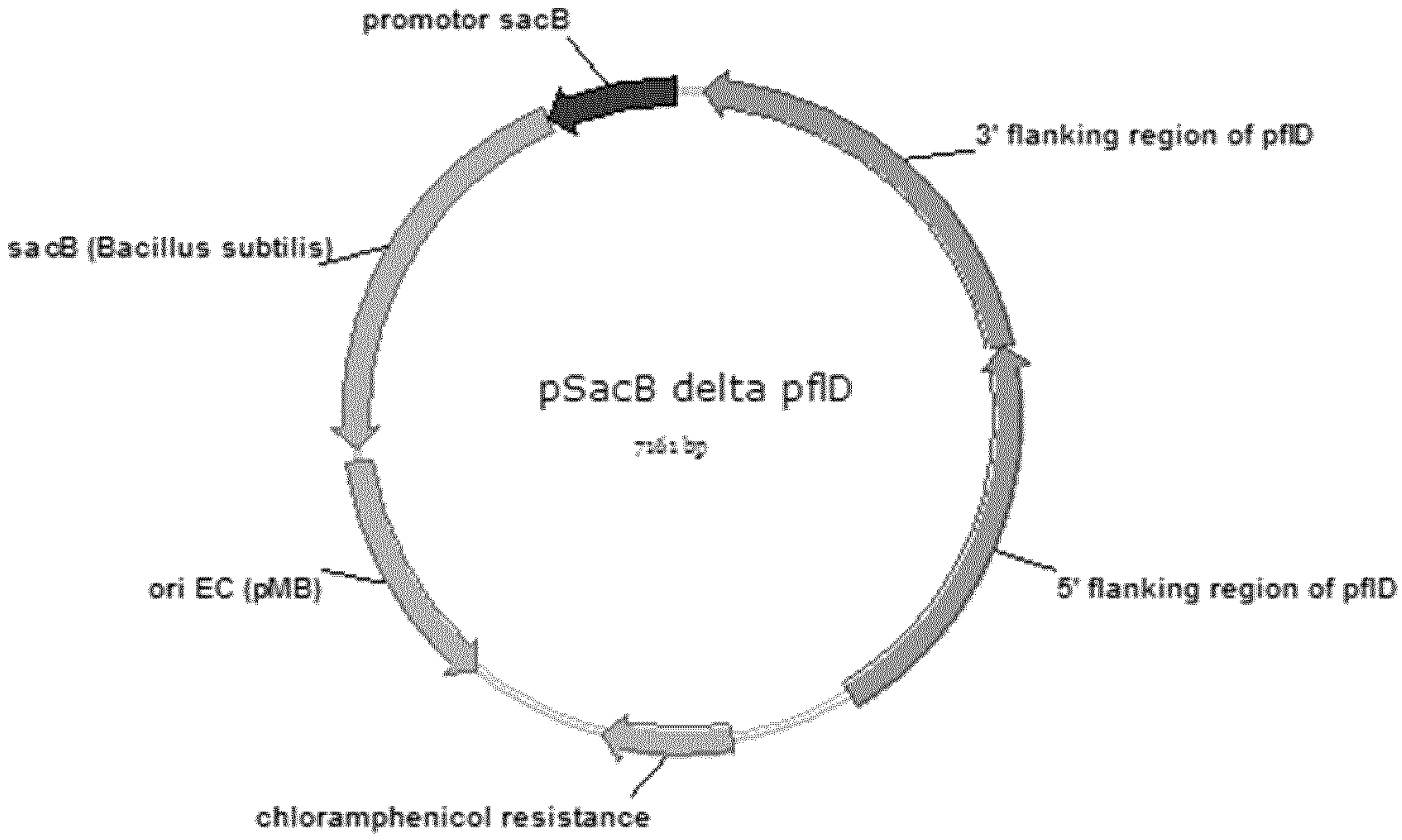
promotor sacB
3' flanking region of pflD
sacB (Bacillus subtilis)
pSacB delta pflD
7161 bp
ori EC (pMB)
5' flanking region of pflD
chloramphenicol resistance

Fig. 4

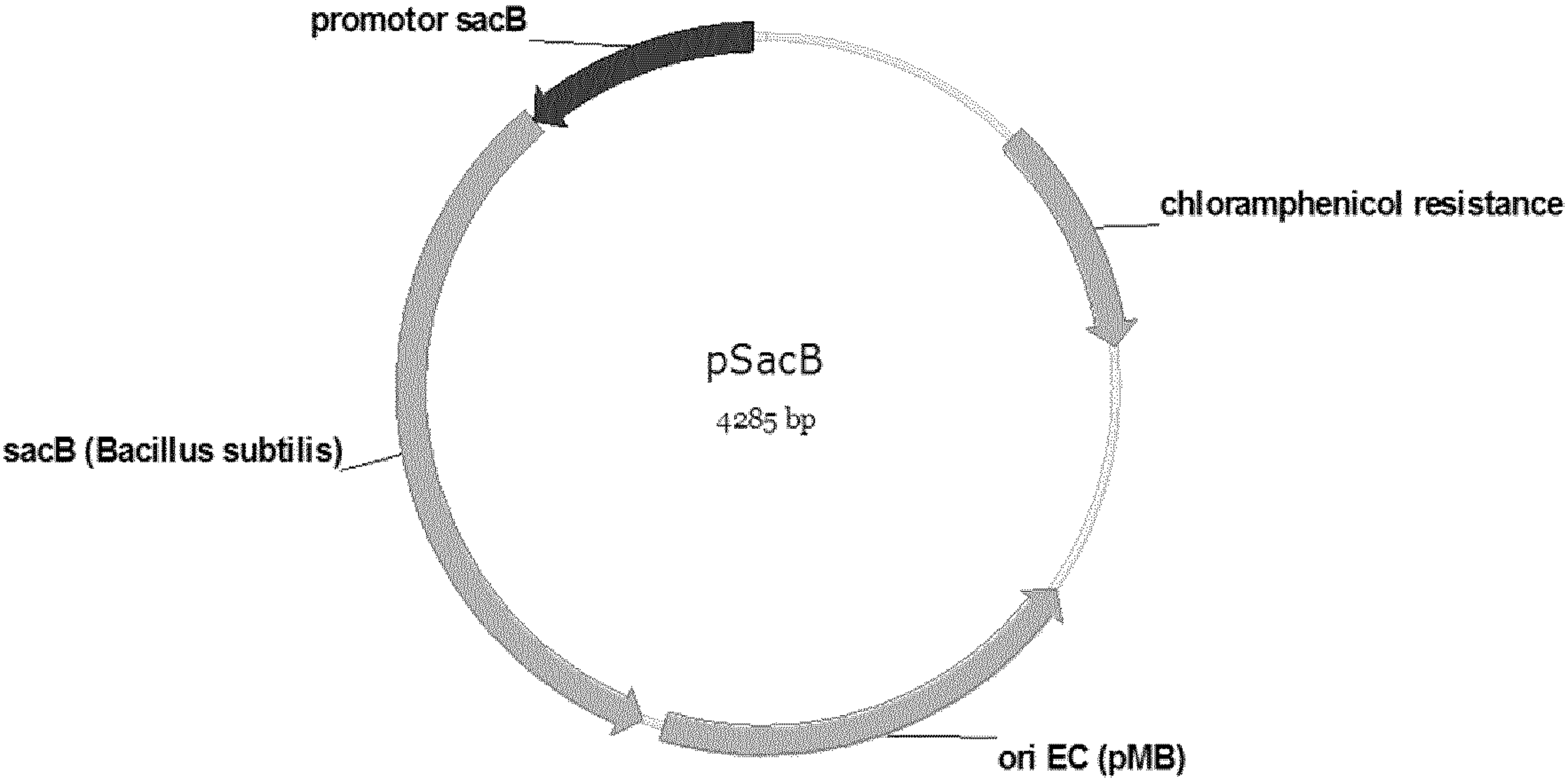
promotor sacB
chloramphenicol resistance
pSacB
4285 bp
sacB (Bacillus subtilis)
ori EC (pMB)

Fig. 1