A process of producing L-lactate as defined herein by feeding carbon dioxide to a culture of a cyanobacterial cell and subjecting said culture to light, wherein said cell is capable of expressing a nucleic acid molecule, wherein the expression of said nucleic acid molecule confers on the cell the ability to convert a glycolytic intermediate into L-lactate and wherein said nucleic acid molecule is under the control of a regulatory system which responds to light or to a change in the concentration of a nutrient in said culture.
IN SITU HYBRIDIZATION METHOD AND BUFFER.

Field of the invention

The invention relates to a process of producing an L-lactate produced in the pathway leading to L-lactate by feeding carbon dioxide to a culture of a cyanobacterial cell and subjecting said culture to light, wherein said cell is capable of expressing a nucleic acid molecule wherein the expression of said nucleic acid molecule confers on said cell the ability to convert a glycolytic intermediate such as pyruvate or glyceraldehyde 3-phosphate into L-lactate and wherein expression of said nucleic acid molecule is under the control of a regulatory system which responds to a change in the concentration of a nutrient in said culture. The invention further relates to a cyanobacterial cell for use in this process.

Background of the invention

Numerous biotechnological processes make use of genetically engineered organisms in order to produce bulk or fine chemicals, proteins or antibiotics. In many cases, increased production has been obtained by improved gene expression and by optimization of growth conditions. In all processes we are aware of, the initial carbon-precursor has been and still is sugar (notably glucose, but many other mono- and polysaccharides are in use) or related organic substrates: solventogenesis (including butanol and ethanol) and organic acid production (e.g. lactic-, citric- or succinic acid) always starts from glucose, which makes it inefficient as the production process uses a high energy initial compound as substrate.

Lactic acid is a naturally occurring organic acid, which has many applications, e.g. it can be used as an acidulant, preservative in the food industry, pharmaceutical, leather and textile industries, as well as a chemical feedstock (Vijayakumar et al. (2008) Chem. Biochem. Eng. Q 22(2):245-264).

Lactic acid can be produced either via chemical synthesis or via microbial fermentation. Currently, most of the lactic acid is produced via microbial fermentation using lactic acid bacteria, although production using filamentous fungi is also known (Vijayakumar et al. vide supra).
However, there is still a need for an alternative and even improved production process of L-lactate, preferably without the need of expensive or complicated starting materials, which process does not have the drawbacks of existing processes.

Description of the invention

Energy ultimately comes from the sun and this energy drives photosynthetic process in plants and photo autotrophic bacteria. This knowledge has led to new methods for the synthesis of biochemicals. In essence, these processes employ plants and algal species to reduce CO\textsubscript{2} to the level of sugars and cell material. After harvesting, these end products are converted to ethanol by yeast fermentation (in the case of crops) or converted chemically to biofuels (in the case of algae). The overall energy conservation of these methods is highly inefficient and therefore demands large surface areas. In addition, the processes are rather labor-intensive, are demanding with respect to water consumption and affect foodstock prices with adverse consequences for food supplies. A more remotely similar process is based on the conversion of solar energy into hydrogen. Also this process suffers from a severely decreased efficiency.

US 6,699,696 describes a process of producing ethanol by feeding carbon dioxide to a cyanobacterial cell, especially a Synechococcus comprising a nucleic acid molecule encoding an enzyme enabling the cell to convert pyruvate into ethanol, subjecting said cyanobacterial cell to sun energy and collecting ethanol. This system has several drawbacks among others the expression system used is temperature sensitive which demands to adapt the production system for such regulation.

WO 2009/078712 describes a process of producing ethanol, propanol, butanol, acetone, 1,3-propanediol, ethylene or D-lactate and where appropriate intermediary compounds in the pathway leading to any of these organic compounds. The process is carried out by feeding carbon dioxide to a culture of cyanobacterial cells and subjecting the culture to light, wherein the cells are capable of expressing a nucleic acid molecule under the control of a regulatory system which responds to a change in the concentration of a nutrient in the culture which confers on the cell the ability to convert a glycolytic intermediate into the above-mentioned organic compounds and/or into intermediary compounds.
The present invention relates to a scalable process for the production of an organic compound suitable as biochemical for large scale plastic production. The invention combines metabolic properties of photoautotrophic and chemoorganotrophic prokaryotes and is based on the employment of recombinant oxyphototrophs with high rates of conversion of Calvin cycle intermediates to a fermentative end product. Its novelty resides in the fact that its core chemical reactions use CO₂ as the sole carbon-containing precursor and light (preferably sunlight) as the sole energy source to drive CO₂ reduction. Preferably, production is controlled by a nutrient- or light-mediated promoter. Using a nutrient-mediated promoter, production is controlled by a medium component and starts at the most appropriate time, namely at the highest possible cell density. Alternatively, a light-mediated promoter is controlled by light intensity. Whereas in current production processes for biochemicals, organisms are substrate (e.g., crops in ethanol production) or product (e.g., microalgae as biodiesel), here microorganisms are used as highly specialized catalysts for the conversion of CO₂ as substrate to a useful end product. These catalysts can be subjected to optimization strategies through physical- and chemical systems-biology approaches. The biochemical background of the invention is more extensively described in example 1 of WO 2009/078712 (herein incorporated by reference). Each aspect of the invention is more extensively described below.

**Cyanobacteria**

In a first aspect, the invention provides a *Cyanobacterium* capable of expressing a nucleic acid molecule, wherein the expression of said nucleic acid molecule confers on the *Cyanobacterium* the ability to convert a glycolytic intermediate into an L-lactate produced in the pathway leading to L-lactate. Preferably, the nucleic acid molecule is under the control of a regulatory system which responds to a change in the concentration of a nutrient or to light intensity when culturing said *Cyanobacterium.*

In the context of the invention a *Cyanobacterium* or a *cyanobacterial* cell (also known as a blue-green algae) is a photosynthetic unicellular prokaryote. Examples of *Cyanobacteria* include the genera *Aphanocapsa, Anabaena, Nostoc, Oscillatoria, Synechococcus, Gloeocapsa, Agmenellum, Scytonema, Mastigocladus, Arthrospira, Haplosiphon.* A preferred genus is *Synechococcus.* A more preferred species of this genus is a *Synechocystis* species. Even more preferably, the *Synechocystis* is a Pasteur
Culture Collection (PCC) 6083 *Synechocystis*, which is a publicly available strain via ATCC for example. A preferred organism used is the phototrophic *Synechocystis* PCC 6083: this is a fast growing cyanobacterium with no specific nutritional demands. Its physiological traits are well-documented: it is able to survive and grow in a wide range of conditions. For example, *Synechocystis* sp. PCC 6803 can grow in the absence of photosynthesis if a suitable fixed-carbon source such as glucose is provided. Perhaps most significantly, *Synechocystis* sp. PCC 6803 was the first photosynthetic organism for which the entire genome sequence was determined (available on http://www.kazusa.or.jp/cyano/cyano.html). In addition, an efficient gene deletion strategy (Shestakov SV et al, (2002), Photosynthesis Research, 73: 279-284 and Nakamura Y et al, (1999), Nucleic Acids Res. 27:66-68) is available for *Synechocystis* sp. PCC 6803, and this organism is furthermore easily transformable via homologous recombination (Grigirieva GA et al, (1982), FEMS Microbiol. Lett. 13: 367-370).

A *Cyanobacterium* as defined herein is capable of converting a glycolytic intermediate into L-lactate as defined herein. A biochemical background of the *Cyanobacteria* of the invention is given in WO 2009/078712 (see e.g. Example 1 of WO 2009/078712).

A *Cyanobacterium* as defined herein preferably comprises a nucleic acid molecule encoding an enzyme capable of converting a glycolytic intermediate into L-lactate as defined herein. A *Cyanobacterium* is therefore capable of expressing a nucleic acid molecule as defined herein, whereby the expression of a nucleic acid molecule as defined herein confers on the *Cyanobacterium* the ability to convert a glycolytic intermediate into L-lactate as defined herein.

"Converting a glycolytic intermediate into L-lactate" preferably means that detectable amounts of an organic compound are detected in the culture of a *Cyanobacterium* as defined herein cultured in the presence of light and dissolved carbon dioxide and/or bicarbonate ions during at least 1 day using a suitable assay for the organic compound.

A preferred concentration of said dissolved carbon dioxide and/or bicarbonate ions is at least the natural occurring concentration at neutral to alkaline conditions (pH 7 to 8) being approximately 1 mM. A more preferred concentration of carbon dioxide and/or bicarbonate ions is higher than this natural occurring concentration. A preferred method
to increase the carbon dioxide and/or bicarbonate ions in solution is by enrichment with waste carbon dioxide from industrial plants. The concentration of carbon dioxide in the gas that is sparged into the culture broth may be increased from the equivalent of 0.03% (air) up to 0.2%.

L-lactate is produced within the cell and may spontaneously diffuse into the culture broth. A preferred assay for L-lactate is High Performance Liquid Chromatography (HPLC). A detectable amount for L-lactate is preferably at least 0.1 mM under said culture conditions and using said assay. Preferably, a detectable amount is at least 0.2mM, 0.3mM, 0.4mM, or at least 0.5mM.

**L-lactate as organic product**

When an organic product to be produced is L-lactate, preferred nucleic acid molecules code for enzymes capable of converting pyruvate into L-lactate, said enzyme comprise a lactate dehydrogenase. Preferred assays for L-lactate are HPLC and enzymatic assays. A detectable amount by HPLC of L-lactate is preferably at least 0.1 mM under said culture conditions as defined earlier herein and using said assay. A detectable amount by enzymatic assays of L-lactate is preferably at least 0.2 mg/l under said culture conditions as defined earlier herein and using said assay. Therefore, in this preferred embodiment, a *Cyanobacterium* comprises a nucleic acid molecule encoding a L-lactate dehydrogenase, preferably a NAD(P)H-dependent L-lactate dehydrogenase (EC 1.1.1.27; also known as ldh, ldhB; preferably from Lactococcus lactis, more preferably from Lactococcus lactis subsp. lactis MG1363)

Accordingly, this preferred embodiment relates to a *Cyanobacterium* capable of expressing at least one nucleic acid molecule, said nucleic acid molecule being represented by a nucleotide sequence, wherein the expression of this nucleotide sequence confers on the cell the ability to convert the glycolytic intermediate pyruvate into L-lactate:

(a) a nucleotide sequence encoding a L-lactate dehydrogenase, wherein said nucleotide sequence is selected from the group consisting of:
i. nucleotide sequences encoding a L-lactate dehydrogenase, said L-lactate dehydrogenase comprising an amino acid sequence that has at least 40% sequence identity with the amino acid sequence of SEQ ID NO: 2.

ii. nucleotide sequences comprising a nucleotide sequence that has at least 40% sequence identity with the nucleotide sequence of SEQ ID NO: 1.

iii. nucleotide sequences the reverse complementary strand of which hybridizes to a nucleic acid molecule of sequence of (i) or (ii);

iv. nucleotide sequences the sequences of which differs from the sequence of a nucleic acid molecule of (iii) due to the degeneracy of the genetic code.

Each nucleotide sequence or amino acid sequence described herein by virtue of its identity percentage (at least 40%) with a given nucleotide sequence or amino acid sequence respectively has in a further preferred embodiment an identity of at least 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or more, and most preferably 100% identity with the given nucleotide or amino acid sequence respectively. In a preferred embodiment, sequence identity is determined by comparing the whole length of the sequences as identified herein.

Each nucleotide sequence encoding an enzyme as described herein may encode either a prokaryotic or an eukaryotic enzyme, i.e. an enzyme with an amino acid sequence that is identical to that of an enzyme that naturally occurs in a prokaryotic or eukaryotic organism. The present inventors have found that the ability of a particular enzyme or to a combination of particular enzymes as defined herein to confer to a Cyanobacterial cell the ability to convert a glycolytic intermediate into L-lactate does not depend so much on whether the enzyme is of prokaryotic or eukaryotic origin. Rather this depends on the relatedness (identity percentage) of the enzyme amino acid sequence or corresponding nucleotide sequence to that of the corresponding identified SEQ ID NO.

Alternatively or in combination with previous preferred embodiments, the invention relates to a further preferred embodiment, wherein at least one enzyme as defined herein is substantially not sensitive towards oxygen inactivation. "Being substantially not sensitive towards oxygen inactivation" preferably means that when such enzyme is expressed in a Cyanobacterium as described herein and when this Cyanobacterium is
cultured in a process of the invention, significant activity of said enzyme is detectable
using a specific assay known to the skilled person. More preferably, a significant
activity of said enzyme is at least 20% of the activity of the same enzyme expressed in
the same *Cyanobacterium* but cultured in the absence of oxygen. Even more preferably,
at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%,
95% of the activity is detectable. Most preferably, the activity of said enzyme as
expressed in a *Cyanobacterium* as described herein and when this *Cyanobacterium* is
cultured in the process of the invention is identical with the activity of the same enzyme
as expressed in a same *Cyanobacterium* as described herein and when this
*Cyanobacterium* is cultured in the absence of oxygen. This is an advantage of the
present invention that the *Cyanobacterium* hence obtained is preferably used in a
process of the invention wherein oxygen is produced, since it will substantially not
affect the activity of the enzymes used herein.

Alternatively or in combination with previous preferred embodiments, the invention
relates to a further preferred embodiment wherein, a *Cyanobacterium* as defined herein
is a *Cyanobacterium* that has been transformed with a nucleic acid construct
comprising a nucleotide sequence encoding an enzyme as defined above depending on
the organic product to be produced. A nucleic acid construct comprising a nucleic acid
molecule coding for a given enzyme as defined herein will ensure expression of the
given nucleic acid molecule, and of the corresponding enzyme in a *Cyanobacterium*. In
a more preferred embodiment, a nucleic acid construct comprises more than one
nucleic acid molecule, each nucleic acid molecule coding for a given enzyme. In an
even more preferred embodiment, a nucleic acid construct comprises two, three, four
nucleic acid molecules, each nucleic acid molecule coding for a given enzyme. In a
most preferred embodiment, a nucleic acid construct comprises all nucleic acid
molecules needed for the conversion of a glycolytic intermediate into L-lactate, each
nucleic acid molecule coding for a given enzyme. This most preferred embodiment is
illustrated in example 2. In this most preferred embodiment, a nucleic acid construct
comprises an expression cassette, said expression cassette comprising each needed
nucleic acid molecule. Each nucleic acid molecule is operably linked with other nucleic
acid molecule present. Most preferably, a suitable promoter is operably linked with the
expression cassette to ensure expression of the nucleic acid molecule in a *Cyanobacterium* as later defined herein.

To this end, a nucleic acid construct may be constructed as described in e.g. US 6,699,696 or US 4,778,759. A *Cyanobacterium* may comprise a single but preferably comprises multiple copies of each nucleic acid construct. A nucleic acid construct may be maintained on a plasmid which is subject to autonomous replication or it may be maintained on a nucleic acid designed for integration into the host chromosome. Suitable plasmid nucleic acid constructs may e.g. be based on the pBluescript from the company Stratagene or on any other plasmid. Preferably, however, each nucleic acid construct is integrated in one or more copies into the genome of a *cyanobacterial* cell. Integration into a *cyanobacterial* cell's genome may occur at random by illegitimate recombination but preferably a nucleic acid construct is integrated into the *Cyanobacterium* cell's genome by homologous recombination as is well known in the art (US 4,778,759). Homologous recombination occurs preferably at a neutral integration site. A neutral integration site is an integration which is not expected to be necessary for the production process of the invention, i.e. for the growth and/or the production of L-lactate as defined herein. A preferred integration site is the nrt operon as illustrated in the examples (Osanai, T., Imamura, S., Asayama, M., Shirai, M., Suzuki, I., Murata, N., Tanaka, K. (2006) Nitrogen induction of sugar catabolic gene expression in *Synechocystis* sp. PCC 6803. *DNA Research* 13, 185-19). Accordingly, in a more preferred embodiment, a *cyanobacterial* cell of the invention comprises a nucleic acid construct comprising a nucleic acid molecule, said nucleic acid molecule being represented by a nucleotide sequence, said nucleotide sequence being a coding sequence of an enzyme as identified herein. Said *cyanobacterial* cell is capable of expression of these enzymes. In an even more preferred embodiment, a nucleic acid molecule encoding an enzyme is operably linked to a promoter that causes sufficient expression of a corresponding nucleic acid molecule in a *Cyanobacterium* to confer to a *Cyanobacterium* the ability to convert a glycolytic intermediate into L-lactate. In case of an expression cassette as earlier defined herein, a promoter is upstream of the expression cassette. Accordingly, in a further aspect, the invention also encompasses a nucleic acid construct as earlier outlined herein. Preferably, a nucleic acid construct comprises a nucleic acid molecule encoding an enzyme as earlier defined herein. Nucleic acid molecules encoding an enzyme have been all earlier defined herein.
A promoter that could be used to achieve the expression of a nucleic acid molecule coding for an enzyme as defined herein may be not native to a nucleic acid molecule coding for an enzyme to be expressed, i.e. a promoter that is heterologous to the nucleic acid molecule (coding sequence) to which it is operably linked. Although a promoter preferably is heterologous to a coding sequence to which it is operably linked, it is also preferred that a promoter is homologous, i.e. endogenous to a Cyanobacterium. Preferably, a heterologous promoter (to the nucleotide sequence) is capable of producing a higher steady state level of a transcript comprising a coding sequence (or is capable of producing more transcript molecules, i.e. mRNA molecules, per unit of time) than is a promoter that is native to a coding sequence, preferably under conditions where sun light and carbon dioxide are present. A suitable promoter in this context includes both constitutive and inducible natural promoters as well as engineered promoters. A promoter used in a Cyanobacterium cell of the invention may be modified, if desired, to affect its control characteristics. A preferred promoter is a PsbA2, as is further outlined below in the next paragraph.


In a more preferred embodiment, the expression of a nucleic acid molecule is induced when a culture is exposed to higher light intensities such as for example the light intensity of day as compared to the light intensity at night. As exemplified in example 4, this is preferably achieved by using a PsbA2 promoter in a nucleic acid construct comprising a nucleic acid molecule as defined herein. Such promoter is always active at a basal level, hence also under standard low intensity growth light as well as in darkness. During the day (at least irradiance of 250, 260, 270, 280, 290 or 300 µE/mVsec), a Cyanobacterium of the invention will grow and produce L-lactate.
During the night (less irradiance than 100, 90, 80, 70, 60 or 50 µE/mVsec), a Cyanobacterium will not grow and expression of the L-lactate producing enzyme L-lactate dehydrogenase is lowered. When light is present at considerable higher intensities, e.g. at least 250, 260, 270, 280, 290 or 300 µE/mVsec, as commonly used standard growth light intensities, the PsbA2 promoter is induced. As a consequence, in this process there is more production of L-lactate as defined herein if the cells are exposed to high light intensity, i.e. at least 250, 260, 270, 280, 290 or 300 µE/m²/sec. There is a basal production if cells are kept in darkness or at light intensities below 100, 90, 80, 70, 60 or 50 µE/m²/sec. This production process has several advantages compared to production processes under a constitutive promoter only: a) As with a constitutive promoter the expression of a nucleic acid construct comprising a nucleic acid molecule as defined herein is always active and therefore L-lactate will always be formed; and b) The yield of L-lactate will be improved. Although not wishing to be bound by any theory, this might be due to the fact that high light treatment as defined above results in higher expression of the nucleic acid molecule as defined herein, whereas at the same time the availability of high light provides also a higher carbon flux to the central carbon metabolite pool. The skilled person knows how to assess the intensity of light in such a way that the cultures production is optimized regarding light influx and its carbon balance.

encoding the photosystem II D1 protein in Synechocystis sp. PCC6803. Photosynthetic Research 92 315-325). Preferably, the cells are exposed to light intensities above 250 μE/m²/sec for at least 15 minutes, however they may be exposed longer, such as for hours, for days or for weeks. Preferably, the psbA2 promoter as identified in SEQ ID NO:5 is used or a promoter which has at least 80% identity with the sequence as provided in SEQ ID NO:5.

Alternatively or in combination with previous embodiments, a nucleic acid molecule as defined herein is expressed constitutively and is regulated so as to respond to a change in the concentration of a nutrient when culturing said Cyanobacteria of the invention. Preferably, this is achieved by a promoter, more preferrably, the promoter is a SigE controlled promotor of the glyceraldehyde dehydrogenase gene from Synechocystis PCC 6083 as identified in SEQ ID NO:3 (Takashi Osanai, et al, Positive Regulation of Sugar Catabolic Pathways in the Cyanobacterium Synechocystis sp. PCC 6803 by the Group 2 sigma Factor SigE. J. Biol. Chem. (2005) 35: 30653-30659) or a promoter which as at least 80% identity with the sequence as provided in SEQ ID NO:3. This promoter is quite advantageous to be used as outlined below in the next paragraph. Alternatively or in combination with previous preferred embodiments, the invention relates to a further preferred embodiment, wherein the expression of a nucleic acid molecule as defined herein is regulated so as to respond to a change in the concentration of a nutrient such as ammonium (Osanai, T., Imamura, S., Asayama, M., Shirai, M., Suzuki, I., Murata, N., Tanaka, K. (2006) Nitrogen induction of sugar catabolic gene expression in Synechocystis sp. PCC 6803. DNA Research 13, 185-195).

In a more preferred embodiment, the expression of a nucleic acid molecule is induced when ammonium concentration is below a given value. This is preferably achieved by using a SigE promoter in a nucleic acid construct comprising a nucleic acid molecule as defined herein. Such promoter is inactive in a first phase of the process when ammonium is present in a concentration which is approximately above 1 mM. In this first phase, a Cyanobacterium will grow and not produce any L-lactate as defined herein. When the ammonium source, has been used for growth and its concentration is approximately below 1 mM, the SigE promoter is induced. As a consequence, the process is divided in 2 phases, a first phase where cell numbers increase and a second phase of the production process of the invention, which is characterized by the
production of L-lactate as defined herein. This two phased production process has several advantages compared to one phase production processes: a) the growth phase is separated from the production phase and therefore high cell densities can be obtained in a short time b) the yield of L-lactate as defined herein will be improved due to the fact that no carbon flux to growth will occur in the second phase. The skilled person knows how to assess the concentration of a nutrient such as ammonium in the culture.

**Method**

In a second aspect, the invention relates to a process of producing L-lactate as defined herein by feeding carbon dioxide to a culture of a cyanobacterial cell and subjecting said culture to light, wherein said cell is capable of expressing a nucleic acid molecule, wherein the expression of said nucleic acid molecule confer on the cell the ability to convert a glycolytic intermediate into L-lactate and wherein said nucleic acid molecule is under the control of a regulatory system which responds to a change in the concentration of a nutrient in said culture.

A Cyanobacterium, a glycolytic intermediate, L-lactate, a nucleic acid molecule, and a regulatory system have all earlier been defined herein.

In a process of the invention, carbon dioxide is fed to a culture broth of Cyanobacteria. The skilled person knows that the carbon dioxide concentration is dependent from the temperature, the pH and the concentration of carbon dioxide present in the air used. Therefore, this is quite difficult to give an estimation of the concentration of carbon dioxide which is being used. Below, we give estimations of preferred concentrations used. A preferred feeding concentration of carbon dioxide is air enriched to 5% carbon dioxide. A preferred source of carbon dioxide may be the waste gas from an industrial plant.

Usually a process is started with a culture (also named culture broth) of Cyanobacteria having an optical density measured at 660 nm of approximately 0.2 to 2.0 (OD$_{660}$=0.2 to 2) as measured in any conventional spectrophotometer with a measuring path length of 1 cm. Usually the cell number in the culture doubles every 20 hours. A preferred process takes place in a tank with a depth of 30-50 cm exposed to sun light. In a preferred process, the number of cells increases until the source of ammonium is exhausted or below a given value as earlier explained herein, subsequently the production of L-lactate will start. In a preferred embodiment, the light used is natural.
A preferred natural light is sunlight. Daylight (or sunlight) may have an intensity ranged between approximately 500 and approximately 1500 µE in/s cm²/s. In another preferred embodiment, the light used is artificial. Such artificial light may have an intensity ranged between approximately 70 and approximately 800 µEinsteins/m²/s.

Preferably, the cells are continuously under the light conditions as specified herein. However, the cells may also be exposed to high light intensities (such as e.g. daylight/sunlight) as defined elsewhere herein for a certain amount of time, after which the cells are exposed to a lower light intensity as defined elsewhere herein for a certain amount of time, and optionally this cycle is repeated. In a preferred embodiment, the cycle is the day/night cycle.

In a preferred process, L-lactate is separated from the culture broth. This may be realized continuously with the production process or subsequently to it. Separation may be based on bipolar fractionating electrodialysis, membrane separation and/or precipitation methods. The skilled person will know which separating method is the most appropriate, such as for example as described in US patent 6,280,985, US patent 2,350,370, Vijayakumar et al. (2008) Chem. biochem. Eng. Q 22(2):245-264 or as described in http://www.jurag.dk/Lactic-acid-process-description.pdf.

Nucleic acid molecule and expression vector

In a further aspect, the invention relates to a nucleic acid molecule comprising a nucleotide sequence encoding a L-lactate dehydrogenases defined above and wherein the nucleotide sequence is under the control of a regulatory system which responds to light as is earlier defined herein. Preferably, a nucleotide sequence according to the invention is operably linked to a light-regulated promoter, preferably a psbA2 promoter, more preferably a light-regulated promoter that has at least 80% nucleic acid sequence identity with SEQ ID NO: 5, as further defined above.

The invention also relates to an expression vector comprising a nucleic acid molecule of the invention. Preferably, an expression vector comprises a nucleotide sequence encoding a L-lactate dehydrogenase of the invention, which is operably linked to one or more control sequences, which direct the production of the encoded polypeptide in a cyanobacterium and wherein the nucleotide sequence is under the control of a regulatory system which responds to light as is earlier defined herein. An expression
vector may be seen as a recombinant expression vector. An expression vector may be any vector which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of a nucleotide sequence encoding a polypeptide of the invention in a cyanobacterium.

5

General definitions
Sequence identity and similarity

Sequence identity is herein defined as a relationship between two or more amino acid (polypeptide or protein) sequences or two or more nucleic acid (polynucleotide) sequences, as determined by comparing the sequences. Usually, sequence identities or similarities are compared over the whole length of the sequences compared. In the art, "identity" also means the degree of sequence relatedness between amino acid or nucleic acid sequences, as the case may be, as determined by the match between strings of such sequences. "Similarity" between two amino acid sequences is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide. "Identity" and "similarity" can be readily calculated by various methods, known to those skilled in the art. In a preferred embodiment, sequence identity is determined by comparing the whole length of the sequences as identified herein.

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include e.g. the BestFit, BLASTP, BLASTN, and FASTA (Altschul, S. F. et al, J. Mol. Biol. 215:403-410 (1990), publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al, NCBI NLM NIH Bethesda, MD 20894). A most preferred algorithm used is EMBOSS (http://www.ebi.ac.uk/emboss/align). Preferred parameters for amino acid sequences comparison using EMBOSS are gap open 10.0, gap extend 0.5, Blosum 62 matrix. Preferred parameters for nucleic acid sequences comparison using EMBOSS are gap open 10.0, gap extend 0.5, DNA full matrix (DNA identity matrix).

Optionally, in determining the degree of amino acid similarity, the skilled person may also take into account so-called "conservative" amino acid substitutions, as will be clear to the skilled person. Conservative amino acid substitutions refer to the
interchangeability of residues having similar side chains. For example, a group of
amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and
isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and
threonine; a group of amino acids having amide-containing side chains is asparagine
and glutamine; a group of amino acids having aromatic side chains is phenylalanine,
tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine,
arginine, and histidine; and a group of amino acids having sulphur-containing side
chains is cysteine and methionine. Preferred conservative amino acids substitution
groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-
valine, and asparagine-glutamine. Substitutional variants of the amino acid sequence
disclosed herein are those in which at least one residue in the disclosed sequences has
been removed and a different residue inserted in its place. Preferably, the amino acid
change is conservative. Preferred conservative substitutions for each of the naturally
occurring amino acids are as follows: Ala to ser; Arg to lys; Asn to gin or his; Asp to
glu; Cys to ser or ala; Gin to asn; Glu to asp; Gly to pro; His to asn or gin; Ile to leu or
val; Leu to ile or val; Lys to arg; gin or glu; Met to leu or ile; Phe to met, leu or tyr; Ser
to thr; Thr to ser; Trp to tyr; Tyr to trp or phe; and, Val to ile or leu.

Hybridising nucleic acid sequences

Nucleotide sequences encoding the enzymes expressed in the cell of the invention
or promoters used in the cell of the invention may also be defined by their capability to
hybridise with the nucleotide sequences of SEQ ID NO. 1, 3, or 5, respectively, under
moderate, or preferably under stringent hybridisation conditions. Stringent
hybridisation conditions are herein defined as conditions that allow a nucleic acid
sequence of at least about 25, preferably about 50 nucleotides, 75 or 100 and most
preferably of about 200 or more nucleotides, to hybridise at a temperature of about
65°C in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution
having a comparable ionic strength, and washing at 65°C in a solution comprising
about 0.1 M salt, or less, preferably 0.2 x SSC or any other solution having a
comparable ionic strength. Preferably, the hybridisation is performed overnight, i.e. at
least for 10 hours and preferably washing is performed for at least one hour with at
least two changes of the washing solution. These conditions will usually allow the
specific hybridisation of sequences having about 90% or more sequence identity.
Moderate conditions are herein defined as conditions that allow a nucleic acid sequences of at least 50 nucleotides, preferably of about 200 or more nucleotides, to hybridise at a temperature of about 45°C in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution having a comparable ionic strength, and washing at room temperature in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution having a comparable ionic strength. Preferably, the hybridisation is performed overnight, i.e. at least for 10 hours, and preferably washing is performed for at least one hour with at least two changes of the washing solution. These conditions will usually allow the specific hybridisation of sequences having up to 50% sequence identity. The person skilled in the art will be able to modify these hybridisation conditions in order to specifically identify sequences varying in identity between 50% and 90%.

*Homologous*

The term "homologous" when used to indicate the relation between a given (recombinant) nucleic acid or polypeptide molecule and a given host organism or host cell, is understood to mean that in nature the nucleic acid or polypeptide molecule is produced by a host cell or organisms of the same species, preferably of the same variety or strain. If homologous to a host cell, a nucleic acid sequence encoding a polypeptide will typically be operably linked to another promoter sequence than in its natural environment. When used to indicate the relatedness of two nucleic acid sequences the term "homologous" means that one single-stranded nucleic acid sequence may hybridise to a complementary single-stranded nucleic acid sequence. The degree of hybridization may depend on a number of factors including the amount of identity between the sequences and the hybridization conditions such as temperature and salt concentration as earlier presented. Preferably the region of identity is greater than about 5 bp, more preferably the region of identity is greater than 10 bp. Preferably, two nucleic acid or polypeptides sequences are said to be homologous when they have more than 80% identity.

*Heterologous*

The term "heterologous" when used with respect to a nucleic acid (DNA or RNA) or protein refers to a nucleic acid or protein (also named polypeptide or enzyme) that does
not occur naturally as part of the organism, cell, genome or DNA or RNA sequence in
which it is present, or that is found in a cell or location or locations in the genome or
DNA or RNA sequence that differ from that in which it is found in nature. Heterologous nucleic acids or proteins are not endogenous to the cell into which it is introduced, but has been obtained from another cell or synthetically or recombinantly produced. Generally, though not necessarily, such nucleic acids encode proteins that are not normally produced by the cell in which the DNA is transcribed or expressed. Similarly exogenous RNA encodes for proteins not normally expressed in the cell in which the exogenous RNA is present. Heterologous nucleic acids and proteins may also be referred to as foreign nucleic acids or proteins. Any nucleic acid or protein that one of skill in the art would recognize as heterologous or foreign to the cell in which it is expressed is herein encompassed by the term heterologous nucleic acid or protein. The term heterologous also applies to non-natural combinations of nucleic acid or amino acid sequences, i.e. combinations where at least two of the combined sequences are foreign with respect to each other.

Operably linked

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements (or coding sequences or nucleic acid sequence or nucleic acid molecule) in a functional relationship. A nucleic acid sequence is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the nucleic acid sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

Promoter

As used herein, the term "promoter" refers to a nucleic acid fragment that functions to control the transcription of one or more nucleic acid molecules, located upstream with respect to the direction of transcription of the transcription initiation site of the nucleic acid molecule, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites,
repressor and activator protein binding sites, and any other sequences of nucleotides
known to one of skill in the art to act directly or indirectly to regulate the amount of
transcription from the promoter. A "constitutive" promoter is a promoter that is active
under most environmental and developmental conditions. An "inducible" promoter is a
promoter that is active under environmental or developmental regulation.

Genetic modifications

For overexpression of an enzyme in a host cells of the inventions as described
above, as well as for additional genetic modification of a host cell, preferably
Cyanobacteria, host cells are transformed with the various nucleic acid constructs of
the invention by methods well known in the art. Such methods are e.g. known from
standard handbooks, such as Sambrook and Russel (2001) "Molecular Cloning: A
Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor
Laboratory Press, or F. Ausubel et al, eds., "Current protocols in molecular biology",
transformation and genetic modification of cyanobacteria! cells are known from e.g.
US 6,699,696 or US 4,778,759.

A promoter for use in a nucleic acid construct for overexpression of an enzyme in
a cyanobacteria! cell of the invention has been described above. Optionally, a
selectable marker may be present in a nucleic acid construct. As used herein, the term
"marker" refers to a gene encoding a trait or a phenotype which permits the selection
of, or the screening for, a Cyanobacteria! cell containing the marker. A marker gene
may be an antibiotic resistance gene whereby the appropriate antibiotic can be used to
select for transformed cells from among cells that are not transformed. Preferably
however, a non-antibiotic resistance marker is used, such as an auxotrophic marker
(URA3, TRP1, LEU2). In a preferred embodiment, a Cyanobacteria! cell transformed
with a nucleic acid construct is marker gene free. Methods for constructing
recombinant marker gene free microbial host cells are disclosed in EP-A-0 635 574 and
are based on the use of bidirectional markers. Alternatively, a screenable marker such
as Green Fluorescent Protein, lacL, luciferase, chloramphenicol acetyltransferase, beta-
glucuronidase may be incorporated into a nucleic acid construct of the invention
allowing to screen for transformed cells.
Optional further elements that may be present in a nucleic acid construct of the invention include, but are not limited to, one or more leader sequences, enhancers, integration factors, and/or reporter genes, intron sequences, centromers, telomers and/or matrix attachment (MAR) sequences. A nucleic acid construct of the invention can be provided in a manner known per se, which generally involves techniques such as restricting and linking nucleic acids/nucleic acid sequences, for which reference is made to the standard handbooks, such as Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press.


In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition the verb "to consist" may be replaced by "to consist essentially of" meaning that a peptide or a composition as defined herein may comprise additional component(s) than the ones specifically identified, said additional component(s) not altering the unique characteristic of the invention. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety. The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Description of the figures
**Figure 1**: pBPSldh. The construct represents the transcriptional coupled approach. HOM1 and HOM2 are the integration platforms to facilitate (double) homologous recombination with the respective sequence in the cyanobacterial genome. KanR resulting in kanamycine resistant is used as positive (antibiotic) marker. The plasmid is based on pBluescript (SK+ II, Stratagene). In SEQ ID NO: 4 the nucleic acid sequence of pBPSldh is given.

**Figure 2**: L-lactate detection in *Synechocystis* *P*$_{p_{sh}A^{2-}::p_{sh}A^{2::ldh::kan}}$ cultures growing in BG-11 depicted on the left Y-axis. OD730 times 100 on the right (log scale) Y-axis.

**Figure 3**: Growth curve of Synechocystis glucose non-tolerant cultures growing in BG-11 supplemented with 10mM TES and 5mM glucose at 37°C.

**Figure 4**: Growth of *Synechocystis* ldh-8 in a 1.8 liter continuous growth system. X-axis indicates time in hours, y-axis the cell concentration in gram/litre. 1 and 2 indicate two biological replicates.

**Examples**

**Example 1**:

Promoter sequence of *psbA2* of *Synechocystis* sp. PCC 6803: (SEQ ID NO: 5)

```
TAATGTATGCCCGACTATT GCTTAAACTGACTGACCACTGACCTTAAGAGT
AATGGCGTGCAAGGCCCAGTGATCAATTTCATTATTTTTCATTATTTCATCT
CCATTGTCCCTGAATCAGTTGTGTCGCCCCTCTACACAGCCCAGAACTAT
GGTAAAGGCGCACGAAAAACCGCCAGGTAAACTCTTCTCAACAGCCCAGAACAT
TGTAACCCGACGAAAACCGCCACGGTAAACTCTTCTCAACAGCCCAGAACAT
CCGCCTTCGTGTATATCTTCGAATACGCTGACGTTTACATCTTGACGACGATT
GAACGACGCACGACGACGACGACGACGACGACGACGACGACGACTTTACAT
```

**Example 2**

Promoter sequence of the unknown DNA segment:

```
TAATGTATGCCCGACTATT GCTTAAACTGACTGACCACTGACCTTAAGAGT
AATGGCGTGCAAGGCCCAGTGATCAATTTCATTATTTTTCATTATTTCATCT
CCATTGTCCCTGAATCAGTTGTGTCGCCCCTCTACACAGCCCAGAACTAT
GGTAAAGGCGCACGAAAAACCGCCAGGTAAACTCTTCTCAACAGCCCAGAACAT
TGTAACCCGACGAAAACCGCCACGGTAAACTCTTCTCAACAGCCCAGAACAT
CCGCCTTCGTGTATATCTTCGAATACGCTGACGTTTACATCTTGACGACGATT
GAACGACGCACGACGACGACGACGACGACGACGACGACGACGACTTTACAT
```
Sequence derived from cyanobase. The promoter sequence is 371bp in length and stops right upstream of the Ribosomal Binding Site (RBS). Primer binding sites are underlined.

Primers used contain sequences for restriction enzymes for cloning purposes:

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<tr>
<td>PpsbA2_R</td>
<td>GTAactgcagccgcgctctacagTAATGTATTGTCGATTTTAGGG</td>
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**Strategy 2. Cloning a gene of interest transcriptional coupled to the psbA2 gene**

Promoter and ORF sequence of psbA2 of Synechocystis sp. PCC 6803 (SEQ ID NO:6; amino acid sequence in SEQ ID NO:7):

```
TAATTGTATGCCCCGACTATTGCTTTAAACTGACTGACCACGTACCTTAAGAG
AATGGCGTGCAAGGCCCAGTGATCAATTTCATTATTTTTCATTATTTTCATCT
CCATTGTCCCTGAAATACGTTTGTGTCGCCCTCTACACAAGGCCCAGAATTAT
GGTAAAGGCCACGAAAAACGCCAGGGTAATACCTTTCACACCCACAAA
CGCCCTCTGTGTTACCGAATGAAACGCAATACGTTACAAAGAGGAAAGTTA
AATGCTCATCTATAAGCTTCGTTGTATATTAACTTCCTGTTACAAAGCTCTT
AACTATATATGTCATCTATAAGCTTCGTTGTATATTAACTTCCTGTTACAA
GAAAGCGCTTCCTTGTGGGAACAGTTTTGTCAGTGGGTGACCTCTACCAAC
AACCGGATATTATGTCGTTTGGGTCTCGGTACCTTGATGATCCACCACCTTT
ACAGCCACCACCTGCTTCATCATTGCCCTTCATCAGCCCGCTCCCCACGGT
CACTCTGTGCTGGTGTACCGCTACCTCCTTCACCCACGCTATGGTGTTCCT
```
Sequence derived from cyanobase. The promoter and gene sequence is 1470bp in length and stops at the stop codon of psbA2. Primer binding sites are underlined. RBS and start codon (ATG) and stop codon (TAA) are bold and underlined.

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<tr>
<td>Hom1Hind_R</td>
<td>TTTAAAGCTTTTTAACCGTGGACAGCA</td>
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L-ldh is derived from *Lactococcus lactis* MG1363 (SEQ ID NO:1 and 2):

```
Atggctgataaaacaagtaaagagtattatcatttgggtgacggtgcctgtaggtcatacatacgcgcccttgaatccacagg
aatgcacaagaattagattagttttgccttttaaaagaaaaaactcaaggggatgcagaagacctttctcatgctcttgccatatta
catcaacctaaagattttactctgcagactactgctagcagctgcctctgctctgctctgcttggagctcactgcaagctgttggc
ccacattccattgaacgatgctgaaatgcaaaaaatggaagcttctggagctcaattgaaagctatcatgatgaagcttttgctaa
agaagaatttgcttctgcagttaaaaactaa
```
**Example 2:**

Biochemical background of the *Cyanobacterium* of the invention

*L-ldh* of the organism *L. lactis* (SEQ ID NO:1) is fused downstream to the transcript of *psbA2* as described in example 1 above. The plasmid was transformed into *Synechocystis* PCC 6803 (freely obtainable, e.g. from Research Group of Aquatic Microbiology (AMB); Prof. dr. Jef Huisman, Institute for Biodiversity and Ecosystem Dynamics; University of Amsterdam, Amsterdam, The Netherlands; or see publications e.g. Hackenberg et al. (2009) *Planta* 230(4): 625-637). Mutant cultures were selected for by growth on agar plates containing 20µg/ml of kanamycin until the genome was fully segregated. This mutant was named *Synechocystis* ldh-8. A scratch of mutant culture was inoculated in BG-1 1 medium supplemented with 10mM TES-buffer-NaOH (pH=8.0) and 10ug/ml kanamycine and grown to stationary phase within several days (OD of 1.5). An aliquot of the initial culture was used to inoculate 100ml BG-1 1 supplemented with 10mM TES-buffer-NaOH (pH=8.0) and with 10µg/ml kanamycine to an OD of 0.1. The culture was incubated at low light intensity (~40µE), 30°C and shaking at 10Orpm. On average every second day 1ml of culture was collected, processed and L-lactate was determined in 100µl the cultures supernatant with an enzymatic assay provided by Megazyme (Megazyme International Ireland Ltd. Ireland). With the help of standard concentrations of L-lactate the concentration of L-lactate in the culture was determined (Figure 2). In conclusion, L-lactate production increases in time (at least up to 30 days) at a rate of more than 20 µmol (gr [dw])⁻¹ h⁻¹.

**Example (3)**

Resistance to lactic acid of *Synechocystis* PCC 6803

The culture was grown in 100ml BG-1 1 supplemented with 10mM TES-buffer-NaOH (pH=8.0) and with 10µg/ml kanamycine to an OD of 0.1. The culture was incubated at
low light intensity (~40 µE), 30°C and shaking at 100rpm. It was clearly shown (Figure 3) that up to a concentration of 50mM L-lactic acid cultures are not affected with respect to growth-rate.

Example (4)

Lactate production under control of a psbA promoter in Synechocystis PCC 6803 in a continuous growth fermentor.

The lactate producing Synechocystis PCC 6803 mutant ldh-8 was grown in a continuous culture with a dilution rate of 0.018 in BG-11 medium with 10mM NaN0₃, 50mM NaC0₃ and 20mM TES buffer. The culture was mixed by air bubbling with 1% added C0₂, and illuminated with continuous white light from a LED-light source at an intensity of ~450 µE. Lactate concentrations were determined with the enzymatic L-lactate assay kit from Megazyme (see Fig 4).

Duplicate samples were taken after 300 hours and washed in BG-11 medium to remove lactate. Lactate production was monitored in batch cultures of 100ml with a cell density of 0.33 g/L for 5 hours at a light intensity of 150 µE.

Duplicate samples were also taken after 600 hours and the lactate concentration was determined directly from chemostat. On average the lactate concentration in chemostat was 647 µM, this gives a lactate flux of 647*0.018/0.36=32.3 µmol · (gr [dw])⁻¹ · h⁻¹. This shows a constant production of L-lactate at a rate of 1 mg/l/hour during at least 3 weeks.
Claims

1. A process of producing L-lactate by feeding carbon dioxide to a culture of a cyanobacterial cells and subjecting said culture to light, wherein said cell is capable of expressing a nucleic acid molecule, wherein the expression of said nucleic acid molecule confers on the cell the ability to convert a glycolytic intermediate into L-lactate.

2. A process according to claim 1, wherein said nucleic acid molecule is under the control of a regulatory system which responds to light intensity or to a change in the concentration of a nutrient of said culture.

3. A process according to claim 1 or claim 2, wherein said enzyme is substantially not sensitive towards oxygen inactivation.

4. A process according to any one of the preceding claims, wherein the nucleic acid molecule codes for an enzyme capable of converting pyruvate to L-lactate, preferably for a L-lactate dehydrogenase, more preferably for a NAD(P)H-dependent L-lactate dehydrogenase.

5. A process according to claim 4, wherein the nucleic acid molecule comprises a nucleotide sequence encoding a L-lactate dehydrogenase, wherein said nucleotide sequence is selected from the group consisting of:

   i. nucleotide sequences encoding a L-lactate dehydrogenase, said L-lactate dehydrogenase comprising an amino acid sequence that has at least 40% sequence identity with the amino acid sequence of SEQ ID NO:2;
   ii. nucleotide sequences comprising a nucleotide sequence that has at least 40% sequence identity with the nucleotide sequence of SEQ ID NO: 1;
   iii. nucleotide sequences the reverse complementary strand of which hybridizes to a nucleic acid molecule of sequence of (i) or (ii); and
   iv. nucleotide sequences the sequences of which differs from the sequence of a nucleic acid molecule of (iii) due to the degeneracy of the genetic code.
6. A process according to any one of the preceding claims, wherein the nucleic acid molecule comprised in the cell is integrated into its genome, preferably via homologous recombination and/or wherein the cyanobacterial cell is derived from a *Synechocystis* cell, preferably a *Synechocystis* PCC 6083 cell.

7. A process according to any one of the preceding claims, wherein the nucleic acid molecule is under the control of a light-regulated promoter, preferably a psbA2 promoter, more preferably a light-regulated promoter that has at least 80% nucleic acid sequence identity with SEQ ID NO: 5.

8. A process according to any one of claims 1-7, wherein the nucleic acid molecule is under the control of a nutrient-regulated promoter, preferably a SigE promoter, more preferably a nutrient-regulated promoter that has at least 80% nucleic acid sequence identity with SEQ ID NO:3.

9. A process according to any one of claims 1-8, wherein L-lactate is separated from the culture.

10. A process according to any one of the preceding claims, wherein the glycolytic intermediate is pyruvate.

11. A nucleic acid molecule comprising a nucleotide sequence encoding a L-lactate dehydrogenase, wherein said nucleotide sequence is selected from the group consisting of:

   i. nucleotide sequences encoding a L-lactate dehydrogenase, said L-lactate dehydrogenase comprising an amino acid sequence that has at least 40% sequence identity with the amino acid sequence of SEQ ID NO:2;

   ii. nucleotide sequences comprising a nucleotide sequence that has at least 40% sequence identity with the nucleotide sequence of SEQ ID NO:1;

   iii. nucleotide sequences the reverse complementary strand of which hybridizes to a nucleic acid molecule of sequence of (i) or (ii); and
iv. nucleotide sequences the sequences of which differs from the sequence of a nucleic acid molecule of (iii) due to the degeneracy of the genetic code; and wherein the nucleotide sequence is under the control of a regulatory system which responds to light or to a change in the concentration of a nutrient of said culture.

12. A nucleic acid molecule according to claim 11, wherein the nucleotide sequence is operably linked to
i) a light-regulated promoter, preferably a psbA2 promoter, more preferably a light-regulated promoter that has at least 80% nucleic acid sequence identity with SEQ ID NO: 5; or,
ii) wherein the nucleotide sequence is operably linked to a nutrient-regulated promoter, preferably a SigE promoter, more preferably a nutrient-regulated promoter that has at least 80% nucleic acid sequence identity with SEQ ID NO: 3.

13. An expression vector comprising a nucleic acid molecule as defined in claim 11 or claim 12.

14. A *Cyanobacterium* capable of expressing a nucleic acid molecule according to claim 11 or 12, or comprising an expression vector according to claim 13, wherein the expression of said nucleic acid molecule confers on the *Cyanobacterium* the ability to convert a glycolytic intermediate into L-lactate and wherein the nucleic acid molecule is under the control of a regulatory system which responds to light intensity or to a change in the concentration of a nutrient when culturing said *Cyanobacteria*.

15. A *Cyanobacterium* according to claim 14, wherein the glycolytic intermediate, is pyruvate.

16. A *Cyanobacterium* according to claim 14 or 15, wherein the nucleic acid molecule comprised in the *Cyanobacteria* is integrated into its genome, preferably via homologous recombination and/or wherein the *Cyanobacterium* is derived from a *Synechocystis*, preferably a *Synechocystis* PCC 6083.
Fig 3

![Graph showing the relationship between log of OD730 and time in hours for different concentrations (0 μM, 100 μM, 1 mM, 5 mM, 10 mM, 50 mM).]
Fig 4
## A. CLASSIFICATION OF SUBJECT MATTER

INV. C12P7/56 C12P1/04 C12N9/04 C12N15/74

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12N

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 practical, search terms used)
EPO-Internal , BIOSIS , EMBASE, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:
  - "X" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  - "Y" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  - "Z" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier document but published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search: 8 July 2010

Date of mailing of the international search report: 30/07/2010

Name and mailing address of the ISA:
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel: (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer
Brenz Verca, Stefano
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