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(54) Titre : BACTERIE DEINOCOCCUS ET SES METHODES D'UTILISATION POUR LA BIOCONVERSION DE
 SUBSTRATS OU LA PRODUCTION DE COMPOSES
 (54) Title: DEINOCOCCUS BACTERIUM AND METHODS OF USE THEREOF FOR BIOCONVERSION OF
 SUBSTRATES OR PRODUCTION OF COMPOUNDS

(57) **Abrégé/Abstract:**

The present invention relates to Deinococcus bacteria and the uses thereof. The invention particularly relates to Deinococcus bacteria having a metabolic pathway ratio between Pentose phosphate and glycolysis greater than 0.5 and less than 5, and their uses in the chemical, pharmaceutical or agro-chemical industries, e.g., for producing compounds of industrial interest such as bioconversion of aliphatic compounds into dicarboxylic acids.

Abstract:

The present invention relates to *Deinococcus* bacteria and the uses thereof. The invention particularly relates to *Deinococcus* bacteria having a metabolic pathway ratio between Pentose phosphate and glycolysis greater than 0.5 and less than 5, and their uses in the chemical, pharmaceutical or agro-chemical industries, e.g., for producing compounds of industrial interest such as bioconversion of aliphatic compounds into dicarboxylic acids.

DEINOCOCCUS BACTERIUM AND METHODS OF USE THEREOF FOR BIOCONVERSION OF SUBSTRATES OR PRODUCTION OF COMPOUNDS

The present invention relates to novel bacteria and the uses thereof. The invention particularly relates to bacteria having a particular metabolic profile and their uses in the chemical, pharmaceutical or agro-chemical industries, e.g., for producing metabolites or drugs of industrial interest.

BACKGROUND

Glucose is the major source of energy in cells. Bacteria typically use two metabolic pathways to catabolize glucose: the glycolysis and, to a lesser extent, the pentose phosphate pathway. Glycolysis converts glucose into pyruvate, and the free energy released is used to form ATP (adenosine triphosphate) and NADH (reduced nicotinamide adenine dinucleotide). The pentose phosphate pathway utilizes glucose to generate various substrates, and produces NADPH (reduced nicotinamide adenine dinucleotide phosphate) and pentoses. More specifically, the pentose phosphate pathway generates NADPH from NADP, in conjunction with the reduction of glucose-6-phosphate into ribulose 5-phosphate.

Although bacteria utilize mainly glycolysis to catabolise glucose, NADPH, which is produced during the pentose phosphate pathway, has a key role in biological reactions that lead to industrially useful compounds. Indeed, cells use NADPH as reducing equivalents for many biosynthetic and oxidation-reduction reactions involved in the protection against the toxicity of reactive oxygen species (ROS), allowing the regeneration of reduced glutathione. NADPH is also used in several important anabolic pathways, such as amino acids synthesis (e.g., arginine, proline, isoleucine, methionine, lysine), vitamin synthesis (e.g., pantothenate, phyloquinone, tocopherol), polyol synthesis (e.g., xylitol), isoprenoid synthesis, fatty acid synthesis, including polyunsaturated fatty acids, as well as in the synthesis of other high added-value substances. In addition, NADPH is the source of reducing equivalents for cytochrome P450 hydroxylation of aromatic compounds, steroids, alcohols and drugs.

Because of the involvement of NADPH in the capacity of cells to conduct important enzymatic reactions, recombinant approaches have been proposed in the art to provide microorganisms with increased cellular NADPH.

For instance, attempts have been made to limit the activity of enzymes involved in the oxidation of NADPH, and/or to increase the activity of enzymes involved in the reduction of NADP. For example, US patent application 10/577,084 relates to strains of microorganisms having one or more of their NADPH-oxidizing activities limited by a deletion of at least one gene coding for a quinone oxidoreductase or a soluble transhydrogenase.

10 US Patent 5,830,716 relates to a recombinant *Escherichia coli* expressing a nicotinamide dinucleotide transhydrogenase. In this bacterium, increased NADPH levels are produced from NADH.

Such approaches allow the production of modified microorganisms having an increased NADPH/NADP ratio. However, modifying the cell metabolism by deregulating specific genes is not entirely satisfactory. Indeed, the metabolic flux in such cells is not stable and cannot be controlled. Furthermore, the metabolic flux may not be adapted easily because the activity of such recombinant cofactor regeneration system is strictly controlled by and dependent on the robustness of the bacteria physiology. In addition, the production of such recombinantly engineered organisms is time and cost consuming, with repercussions on the production of the compounds of interest.

The over-expression of glucose 6-phosphate dehydrogenase to increase NADPH production has also been considered. However, over-expression of the corresponding coding gene is lethal for the cells.

There is therefore always a need for microorganisms suitable for producing industrially useful compounds. There is also a need for a source of reductive or anti-oxidant activity. There is also a need for improved methods for generating reduced compounds or anti-oxidant compositions.

SUMMARY OF THE INVENTION

The present invention describes the isolation and characterization of bacteria having particular metabolic profiles, which are particularly useful and advantageous for the production of high value compounds by biotransformation. More specifically, the invention reports the isolation and characterization of bacteria with a natural imbalance of their metabolism, which favours the pentose phosphate pathway. These bacteria can be used to produce valuable compounds, and also represent a potent anti-oxidant or reductive material.

10 An object of this invention more specifically relates to a bacterium which has a metabolic pathway ratio between Pentose phosphate and glycolysis greater than 0.5. Preferred bacteria of this invention have a metabolic pathway ratio between Pentose phosphate and glycolysis equal to or greater than 1, more preferably comprised between 1 and 20.

15 In a specific embodiment, the bacterium of the invention belongs to a *Deinococcus* species or a related bacterium.

Another object of the invention relates to a method for selecting or optimizing a bacterium, comprising the following steps:

20 a) providing a sample comprising bacteria;
b) subjecting the sample to a cell-destructing DNA damaging treatment, such as a repeated irradiation treatment;
c) identifying or isolating living or growing bacteria from said treated sample;
d) measuring the metabolic pathway ratio between Pentose phosphate and glycolysis of bacteria of step c); and
25 e) selecting a bacterium of step d) which has a metabolic pathway ratio between Pentose phosphate and glycolysis greater than 0.5, preferably equal to or greater than 1, even more preferably comprised between 1 and 20.

30 In a particular embodiment, the treatment of step b) comprises a repeated UV irradiation, particularly a sequential UV treatment, e.g., a repetition of at least 2, preferably 3 or more irradiations at essentially regular intervals.

The invention further relates to a bacterium as defined above, further comprising at least one recombinant nucleic acid sequence. Preferentially, the at least one recombinant nucleic acid sequence encodes an NADPH-dependent enzyme able to catalyze a reaction of interest, such as an NADPH-dependent Alcohol dehydrogenase or a Cytochrome P450 enzyme.

A further object of the invention relates to the use of at least one nucleic acid sequence of a bacterium as defined above, encoding a NADPH-dependent enzyme, to transform a microorganism, particularly a bacterium or a yeast, to provide a recombinant microorganism with a metabolic pathway ratio between Pentose phosphate and glycolysis greater than 0.5. The invention further relates to such a microorganism.

A further object of the invention relates to a co-culture of at least two distinct microorganisms, wherein at least one of said microorganisms is a bacterium as defined above, and at least one of said microorganisms is a prokaryotic or eukaryotic cell, preferentially a yeast, and wherein said at least two microorganisms are symbiotic to each other.

A further object of the invention relates to a cell-free extract of a bacterium as defined above, preferably selected from a cell supernatant, cell debris, cell walls, and DNA extract.

A further object of the invention relates to the use of a bacterium or extract as defined above, in a bioproduction process.

A further object of the invention relates to the use of a bacterium or extract as defined above, for the production of a compound of interest, particularly a metabolite and/or drug.

A further object of the invention relates to the use of a bacterium or extract as defined above, for the bioconversion of a substrate into a substance of interest.

A further object of the invention relates to the use of a bacterium or extract thereof as defined above, for the production of a reduced or anti-oxidant molecule or composition.

A further object of the invention relates to a process for the bioproduction of a compound
5 of interest, the process comprising producing the compound with a bacterium of the invention or an extract thereof and, optionally, recovering the compound. In a particular embodiment, the process comprises exposing a substrate to such a bacterium or extract, the substrate being converted by said bacterium or extract into a compound of interest. In another embodiment, the process comprises exposing the bacterium or extract to a carbon
10 source, the bacterium or extract producing the compound by fermentation of said carbon source.

The compound of interest may be any organic compound such as a protein, peptide, lipid, nucleic acid, organic acid, alcohol, amino acid, etc. The compound may be a drug,
15 metabolite, or fine chemical product such as biofuel, bioplastic, dye, etc. The compound may be in purified form, or a mixed preparation.

In this regard, a further object of the invention relates to a method for the production of a metabolite and/or drug of interest, the method comprising culturing a bacterium as defined
20 above which produces said metabolite and/or drug in an appropriate culture medium, and recovering the metabolite and/or drug of interest from the culture medium.

A further object of the invention relates to a method for the bioconversion of a substrate into a substance of interest, the method comprising culturing a bacterium as defined above
25 having the bioconversion activity in an appropriate culture medium comprising a source of carbon and the substrate under conditions allowing bioconversion, and recovering the substance of interest from the culture medium.

A further object of the invention relates to a method for the production of a reduced
30 molecule of interest, the method comprising culturing a bacterium as defined above which produces said reduced molecule in an appropriate culture medium comprising a source of carbon, and recovering the reduced molecule of interest from the culture medium. Such

reduced molecules can be used as an active ingredient for the preparation of a dietary supplement and/or for the preparation of a cosmetic composition with antioxidant properties.

A further object of the invention is a dietary supplement or a cosmetic composition comprising a bacterium of the invention or an extract thereof.

A further object of the invention resides in a composition comprising a bacterium of the invention or an extract thereof, for use as an anti-oxidant.

A further object of the invention resides in a *Deinococcus* bacterium having a metabolic pathway ratio between Pentose phosphate and glycolysis greater than 0.5 and equal or lower than 5.

LEGEND TO THE FIGURES

Figure 1: Schematic representation of the pentose phosphate and the glycolysis pathways;
Figure 2: Schematic representation of the main metabolic flux ratios in two strains of *Deinococcus*, showing that the flux through the oxidative pentose phosphate pathway in both strains is substantially high (superior to 1). Similarly, the anaplerotic flux from pyruvate to oxaloacetate and malic enzyme flux from malate to pyruvate is high in both strains.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates, generally, to bacteria having a permanent and high metabolic flux through the oxidative pentose phosphate pathway, as well as to their uses for producing substances with NADPH-consuming biosynthesis routes. The bacteria according to the invention can be used in NADPH-consuming biotransformation processes, including fermentation and bioconversion processes. The present invention also concerns methods for the preparation of substances by biotransformation, through growth in an appropriate medium of a bacterium according to the invention. The invention further concerns recombinant bacteria, which are optimized to include genetic elements necessary for the preparation of such substances.

Definitions

In the context of the invention, the term “*Deinococcus*” bacteria includes wild type or natural variant strains of *Deinococcus*, e.g., strains obtained through accelerated evolution, by DNA-shuffling technologies, or recombinant strains obtained by insertion of eukaryotic, 5 prokaryotic and/or synthetic nucleic acid(s). *Deinococcus* bacteria can designate any bacterium of the genus *Deinococcus*, such as without limitation, a *D. geothermalis*, *D. cellulolysiticus*, *D. radiodurans*, *D. proteolyticus*, *D. radiopugnans*, *D. radiophilus*, *D. grandis*, *D. indicus*, *D. frigans*, *D. saxicola*, *D. maricopensis*, *D. marmoris*, *D. deserti*, *D. murrayi*, *D. aeri*, *D. aerolatus*, *D. aerophilus*, *D. aetherius*, *D. alpinitundrae*, *D. altitudinis*, *D. apachensis*, *D. aquaticus*, *D. aquatilis*, *D. aquiradiocola*, *D. aquivivus*, *D. caeni*, *D. claudionis*, *D. ficus*, *D. gobiensis*, *D. hohokamensis*, *D. hopiensis*, *D. misasensis*, *D. navajonensis*, *D. papagonensis*, *D. peraridilitoris*, *D. pimensis*, *D. piscis*, *D. radiomollis*, *D. roseus*, *D. sonorensis*, *D. wulumuqiensis*, *D. xibeiensis*, *D. xinjiangensis*, *D. yavapaiensis* or *D. yunweiensis* bacterium. Preferred *Deinococcus* bacteria are *D. geothermalis*, *D. cellulolysiticus*, *D. deserti*, *D. murrayi*, and *D. radiodurans*. 15

A bacterium “related” to *Deinococcus* designates a bacterium which (i) contains a 16S rDNA which, upon amplification using primers GTTACCCGGAATCACTGGGCGTA (SEQ ID N°1) and GGTATCTACGCATTCCACCGCTA (SEQ ID N°2), generates a 20 fragment of about 158 base pairs and/or (ii) resists a UV treatment of 4 mJ/cm². In a particular embodiment, *Deinococcus*-related bacteria are bacteria having a 16S rDNA molecule which is at least 70%, preferably at least 80% identical in sequence to a *Deinococcus* 16S rDNA sequence.

25

In the context of the invention, the term “*bioproduction*” designates any production process that uses a microorganism, or an extract or isolated part thereof.

In the context of the invention, the term “*fermentation*” designates a process during which a substance is produced by a microorganism from a carbon source. Preferred fermentation 30 processes of the invention involve at least one NADPH-dependent step.

In the context of the invention, the term “*bioconversion*” is the action mediated by a bacterium or an extract thereof for converting a given substrate into a substance of interest. The substrate is usually not assimilated by the bacterium and distinct from the carbon source. Preferred bioconversion processes of the invention include at least one NADPH-
5 dependent step.

The term “*NADPH-dependent*” indicates that a process or reaction involves at least one enzyme whose activation or activity requires or consumes NADPH.

10 A “*gene*” designates any nucleic acid encoding a protein. The term gene encompasses DNA, such as cDNA or gDNA, as well as RNA. The gene may be first prepared by e.g., recombinant, enzymatic and/or chemical techniques, and subsequently replicated in a host cell or an in vitro system. The gene typically comprises an open reading frame encoding a desired protein. The gene may contain additional sequences such as a transcription
15 terminator, a signal peptide, an IRES, an intron, etc. Preferably, the gene does not contain an intron.

The term “*reconstructed*” or “*recombinant*” in relation to a sequence, nucleic acid, or unit in a bacterium, indicates a sequence, nucleic acid or unit which does not exist naturally in
20 the bacterium and has been assembled and/or inserted in said bacterium or an ancestor thereof. The term “*reconstructed*” or “*recombinant*” in relation to a bacterium, indicates a bacterium which does not exist naturally and wherein a sequence, nucleic acid or unit has been assembled and/or inserted in said bacterium or an ancestor thereof.

25 A “*cell-destructing DNA damaging treatment*” indicates a treatment that is sufficient to cause 90% cell death, or more, in a culture of *E. coli* bacteria. Even more preferably, the cell-destructing DNA damaging treatment is a treatment that is sufficient to reduce by at least 2 log the bacterial titer in a culture of *E. coli*. Examples of such treatment include irradiation, preferably repeated and sequential UV irradiation, and/or the use of genotoxic
30 agents. A preferred stress treatment is a UV treatment of between 0.5 and 400 mJ/cm², more preferably of between 1 and 200 mJ/cm², typically between 1 and 100 mJ/cm², applied for a period of time of about 5’’ to 5’. A preferred UV treatment is 4 mJ/cm² for

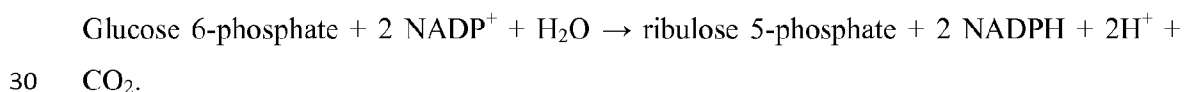
30 seconds, which may be repeated at an interval of between 1 and 8 hours, preferably 3 to 5 hours, and more preferably of about 4 hours. Specific cell stress treatments according to the invention have been described in patent application EP2210935, which is incorporated therein by reference.

- 5 An “*extract of bacteria*” refers to material obtained e.g., following lysis of bacteria. The extract of bacteria comprises at least part of died bacteria, such as DNA, cell wall fragments, enzymes and/or metabolites, etc. The extract may further comprise the culture medium wherein the bacteria have excreted enzymes, metabolites, etc. The extract may contain solely enzyme(s) of particular interest, produced by said bacteria. The extract may
10 be obtained using any method suitable for lysing bacteria. For example, the extract may be obtained by lysing bacterial cells under basic conditions (e.g., by means of an organic or inorganic base). Mechanical methods of lysing bacterial cells may be used too, such as vortex. If required, the extract may be filtered by passage through one or more microfilters or ultrafilters before use, for example in order to remove cell wall fragments from the
15 bacterial extract. The extract may be in liquid or solid form. In a particular embodiment, the extract is lyophilised and conditioned in powder form.

Bacteria with increased Pentose Phosphate Pathway

- 20 The invention stems from the identification of bacteria having a surprisingly high and permanent pentose phosphate pathway flux.

The pentose phosphate pathway (abbreviated as “PP pathway”) refers to a cell process that converts glucose molecules into biochemically useful smaller molecules and generates
25 NADPH. The PP pathway comprises two distinct phases. A first, oxidative phase during which NADPH and ribulose-5-P are generated from glucose. The oxidative phase can be represented by the following chemical reaction:



The second phase, which is non-oxidative, allows the conversion of ribulose-5-phosphate into various substrates, particularly for the construction of nucleotides and nucleic acids.

The NADPH generated during the PP pathway is an important cofactor for reductive biosynthesis reactions within the cells. For example, NADPH provides reducing equivalents for Cytochrome P450 hydroxylation of aromatic compounds, steroids or alcohols, and for various biosynthetic reactions, including fatty acid chain elongation, and lipids or isoprenoid synthesis. Additionally, NADPH provides reducing equivalents for oxidation-reduction involved in protection against the toxicity of reactive oxygen species.

10 The inventors have discovered and isolated a class of bacteria having a surprising and remarkable high PP pathway flux at least during the exponential phase. More particularly, in these bacteria, an important part of glucose is naturally catabolized through the PP pathway, leading to the permanent (e.g., continuous) production of an important amount of NADPH. Bacteria having a flux ratio of pentose phosphate / glycolysis above 0.5 have
15 been selected, and their growth capacity and production ability have been verified by the inventors, confirming their remarkable utility in bioproduction.

An object of the invention therefore resides in a bacterium, wherein said bacterium has a metabolic pathway ratio between Pentose phosphate and glycolysis (herein after PP/G)
20 greater than 0.5.

Such an imbalance of the metabolic pathways is particularly surprising and unprecedented. Indeed, up to now, it was commonly accepted that microorganisms use very preferentially glycolysis for degrading glucose. For example, the innate metabolic pathway ratio Pentose phosphate/glycolysis for *Escherichia coli* is inferior to 0.33 in exponential phase (Macick
25 R. Antoniewicz et al Metab Eng. 2007 May; 9(3): 277-292). In *Saccharomyces cerevisiae*, this ratio is about 0.25 (Paula Jouhten et al BMC Syst Biol. 2008; 2: 60 ; van Winden WAFEMS Yeast Res. 2005 Apr;5(6-7):559-68), and for *Bacillus subtilis* 0.2 (Dauner M Appl Environ Microbiol. 2002 Apr;68(4):1760-71). The invention now discloses a class of
30 bacteria having a PP/G ratio above 0.5. The present invention even discloses the identification of bacteria with a PP/G ratio above 1, 2, 3, 5, 10 or even 15. In such bacteria,

the PP pathway is highly predominant and most glucose is consumed by the pentose phosphate route, the glycolysis being very limited.

Furthermore, in the bacteria of the invention, the high PP/G metabolic ratio is permanent during the exponential phase, i.e., it is not conditioned by specific culture conditions (such as e.g., a high oxidative stress).

The present invention therefore relates to these bacteria, methods for their isolation and culture, as well as to the uses thereof, particularly in the chemical, pharmaceutical and agro-chemical industries, e.g., to produce, by biotransformation, substances with NADPH-consuming biosynthesis routes.

In the context of the invention, a bacterium having a “a metabolic pathway ratio between Pentose phosphate and glycolysis greater than 0.5” designates a bacterium wherein more than one third of the glucose is consumed through the pentose phosphate pathway and the remaining is consumed by glycolysis. When the ratio is equal to or greater than 1, this indicates that at least 50% of the glucose is consumed by said bacterium by the PP pathway.

Preferred bacteria of the invention have a PP/G ratio greater than 2, 2.5, 3, 3.5, 4, or 5.

In a particular embodiment, bacteria of the invention have a metabolic pathway ratio between Pentose phosphate and glycolysis between 1-20, preferably between 2-20. As detailed in the experimental section, the inventors have been able to identify and cultivate bacterium with a PP/G ratio as high as 19, which means bacteria wherein 95% of the glucose consumed is degraded using the pentose phosphate pathway. The inventors show these bacteria are viable, can grow under industrially-compatible culture conditions and do exhibit improved properties for bioproduction processes.

In another particular embodiment, bacteria of the invention have a PP/G ratio comprised between 2 and 10. Specific examples of bacteria of the invention as disclosed in the examples have a PP/G ratio of 1.2; 2.3; 5.6; 9; 19. More particularly, *Deinococcus* strain DGO1 as disclosed in the examples has a PP/G ratio of 1.2. *Deinococcus* strain DGO2 as disclosed in the examples has a PP/G ratio of 19.

- The bacteria of the invention, with increased metabolic pentose phosphate pathway, can be identified and selected using any techniques suitable for measuring metabolic flux distributions. In this regard, an object of the invention also relates to a method for selecting
- 5 or optimizing a bacterium, comprising the following steps:
- a) providing a sample comprising bacteria, preferably a sample comprising uncharacterized bacteria ;
 - d) measuring the metabolic pathway ratio between Pentose phosphate and glycolysis of bacteria in the sample; and
 - 10 e) selecting a bacterium of step d) which has a metabolic pathway ratio between Pentose phosphate and glycolysis greater than 0.5.

The metabolic pathway ratio can be measured by measurement of isotope incorporation into intracellular pools of specific metabolites, preferentially of low abundant metabolites.

15

For example, the PP/G ratio can be evaluated by measuring isotope incorporation into a metabolite characteristic of the PP or G pathway. The % incorporation obtained is directly correlated to the % usage of the corresponding metabolic pathway.

- 20 A specific example of a suitable method is based on a measure of isotope incorporation into glyceraldehyde-3-phosphate (G3P), or a derivative thereof such as serine (see the experimental section, as well as e.g., *Krömer et al. Australian Biochemist, Vol 40 No 3 December 2009*). Indeed, the C₁ carbon atom of glucose is transferred to G3P during glycolysis while, in the first step of the PP pathway, C₁ of glucose-6-phosphate is lost to
- 25 CO₂. Accordingly, no label is retained if glucose-6-phosphate, labeled at C₁ position, is catabolized via PP pathway, while the label is retained through glycolysis, and one mole labeled glucose-6-phosphate gives one mole labeled glyceraldehyde-3-phosphate.

Accordingly, in a particular embodiment, step d) of the process comprises:

- 30
- Culturing the bacteria in a culture medium comprising glucose labeled at C₁ position as a source of carbon, under conditions allowing the growth of bacteria;
 - Recovering and lysing the bacteria;

- Measuring the label incorporation in glyceraldehyde-3-phosphate or a derivative thereof, and using such incorporation to calculate the PP/G ratio.

The label used is typically a radiolabel, such as e.g., ^{13}C .

Further techniques to calculate the PP/G ratio include mass spectrometry, to measure
5 specific metabolites or fluxes, or proteomics.

In an embodiment of the invention, the bacteria are obtained or selected by a process comprising:

- 10 a) providing a sample comprising bacteria, particularly a sample comprising uncharacterized bacteria;
- b) subjecting the sample to a cell-destructing DNA damaging treatment, such as a repeated irradiation treatment;
- c) identifying or isolating living or growing bacteria from said treated sample;
- d) measuring the metabolic pathway ratio between Pentose phosphate and
15 glycolysis of such bacteria, for example by using the ^{13}C metabolic flux analysis as described above; and
- e) selecting bacteria which have a metabolic pathway ratio between Pentose phosphate and glycolysis greater than 0.5, preferably greater than 1.

20 The starting sample may comprise bacteria of any genus or species, in particular uncharacterized or unknown bacteria. The repeated irradiation treatment is typically a UV treatment of between 0.5 and 400 mJ/cm², more preferably of between 1 and 200 mJ/cm², typically between 1 and 100 mJ/cm², applied for a period of time of about 5'' to 5', which may be repeated at an interval of between 1 and 8 hours, preferably 3 to 5 hours, and more
25 preferably of about 4 hours. A preferred UV treatment is 4 mJ/cm² for 30 seconds, repeated at an interval of 4 hours.

The invention also relates to bacteria identified or obtainable by the above methods. As shown in the examples, preferred bacteria of the invention are *Deinococcus* or related
30 bacteria. *Deinococcus* is a gram positive bacterium that was isolated in 1956 by Anderson and collaborators. This extremophile organism is resistant to DNA damage by UV and ionizing radiations or by cross-linking agent (mitomycin C) and is tolerant to desiccation.

WO2009/063079 shows that *Deinococcus* bacteria can resist to solvents and transform biomass to generate ethanol. WO2010/130806 further discloses recombinant *Deinococcus* strains wherein ethanol biosynthesis genes have been inserted. The invention now shows that *Deinococcus* or related bacteria may be isolated, having a permanent ratio PP/G of at least 0.5. These bacteria represent highly valuable biocatalyst for bioproduction processes, as well as for the production of anti-oxidant compositions.

The invention also relates to a method conducting a bioproduction process, the method comprising testing the PP/G ratio of the microorganism used to verify the ratio is equal to or greater than 0.5.

The bacteria of the invention present a substantial advantage over the prior art in that they have a natural elevated PP/G ratio. As mentioned above, some bacteria may have been engineered to increase their NADPH production. However, these bacteria contain inserted nucleic acids, which may confer instability or non-compliance with regulatory guidelines. The bacteria of the invention have a natural and permanent elevated PP/G ratio, and such permanent ratio is not caused by expression of a recombinant nucleic acid. The elevated ratio is permanent in that it is not subject to specific culture or stress conditions. While the ratio may vary in the cells of the invention depending on certain conditions, the basal ratio remains greater than 0.5.

The bacteria of the invention can be grown in specific and/or optimized culture media. Preferentially, the culture medium contains at least lignin, cellulose, hemi-cellulose, starch, xylane, polysaccharide, xylose, sucrose, glucose, galactose, lactose, molasses, or derivatives thereof, as carbon source. Starch refers to a carbohydrate consisting of a large number of glucose units joined together by 1-4 and 1-6 glycosidic bonds. Starch is an energy storage molecule accumulated by many plants and bacteria, and starch molecules arrange themselves in the plant in semi-crystalline granules. A simple source of carbon that is especially preferred is glucose.

The culture medium can also contain one or more substances (e.g. amino acids, vitamins or mineral salts) that favour the growth of the bacteria and/or the production/bioconversion of

substances of interest. In the case of bioconversion, the process also includes the addition to the appropriate culture medium of the substrate that is to be converted. The optimized conditions to favour the growth of the bacteria are set by those skilled in the art. In the same way, those skilled in the art know the nature of the substrates and/or of the carbone
5 sources required in the culture medium to obtain the desired substances. These requirements are documented and can be easily implemented by those skilled in the art.

For example, manganese can be added to the culture medium for improving the incorporation of xylose and galactose in glycoproteins. In addition Manganese acts as a catalyst and cofactor in many enzymatic processes involved in the synthesis of fatty acids
10 and cholesterol. Otherwise, by depleting the culture medium of manganese, it is possible to decrease further the glycolysis. Indeed, Manganese is involved in the transfer of high energy phosphate groups by phosphotransferase enzymes, which are involved in several steps of glycolysis.

A particular object of the invention resides in a composition comprising a culture of
15 bacteria as defined above. In a preferred embodiment, the composition is devoid of cell having a PP/G ratio below 0.5.

Bioproduction Process

Because of their potent metabolic profile, the bacteria of the invention can be used
20 efficiently in bioproduction processes, for producing substances of interest, such as metabolites, drugs, reduced molecules, or anti-oxidant molecules or compositions.

In this regard, an object of the invention resides in a bioproduction process for the production of a molecule, wherein the process utilizes a bacterium having a PP/G ratio of at least 0.5, or an extract thereof.

25 A further object of the invention relates to a process for the bioproduction of a compound, the process comprising producing the compound with a bacterium of the invention or an extract thereof and, optionally, recovering the compound. In a particular embodiment, the process comprises exposing a substrate to such a bacterium or extract, the substrate being converted by said bacterium or extract into a compound of interest. In another

embodiment, the process comprises exposing the bacterium or extract thereof to a carbon source, the bacterium or extract producing the compound by fermentation of said carbon source.

- 5 The compound of interest may be any organic compound such as a protein, peptide, lipid, nucleic acid, organic acid, alcohol, amino acid, etc. The compound may be a drug, metabolite, or fine chemical product. The compound may be in purified form, or a mixed preparation.
- 10 In this regard, a further object of the invention relates to a method for the production of a metabolite and/or drug of interest, the method comprising culturing a bacterium as defined above which produces said metabolite and/or drug in an appropriate culture medium, and recovering the metabolite and/or drug of interest from the culture medium. The process is preferably an NADPH dependent process.

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- A further object of the invention relates to a method for the bioconversion of a substrate into a substance of interest, the method comprising culturing a bacterium as defined above having the bioconversion activity in an appropriate culture medium comprising a source of carbon and the substrate under conditions allowing bioconversion, and recovering the
20 substance of interest from the culture medium. The process is preferably an NADPH dependent process.

- A further object of the invention relates to a method for the production of a reduced
25 molecule of interest, the method comprising culturing a bacterium as defined above which produces said reduced molecule in an appropriate culture medium comprising a source of carbon, and recovering the reduced molecule of interest from the culture medium. Such reduced molecules can be used as an active ingredient for the preparation of a dietary supplement and/or for the preparation of a cosmetic composition with antioxidant properties. The process is preferably an NADPH dependent process.

30

The term “metabolite” designates all possible intermediate molecules generated during a fermentation process, including but not limited to chemical products of industrial interest, such polyols, alcohols, dicarboxylic acids, amino acids, vitamins, sterols, lipids including isoprenoids, flavonoids, fatty acids, and building blocks. The metabolites may also have utility in cosmetics or agriculture, such as pigments, insecticides, pesticides, chemical-degrading compounds, etc.

The term “drug” designates, generally, any compound having a biological activity. This term includes any protein, polypeptide, peptide, chemical compound, lipid, saccharide, etc., having a biological activity which may be used in the human or veterinary pharmaceutical industry. Examples of such drugs include, without limitation, antibiotics, hormones, bacteriostatic compounds, anti-metabolite agents, anti-oxidant agents, chemotherapeutic compounds, anti-fungal agents, anti-viral compounds, cytokine-activity compounds, cell-growth factors, vaccines and adjuvants.

The term “reduced molecule” or “reduced agent” designates, generally, a molecule capable of inhibiting the oxidation of other molecules by promoting its own oxidation (i.e., it donates an electron to another compound in a reduction-oxidation reaction) in response to an oxidative insult. Such reduced molecules present an anti-oxidative activity suitable for reducing the production of reactive oxygen species (ROS). Because ROS can interact with a large number of molecules including other small inorganic molecules as well as DNA, proteins, lipids, carbohydrates and nucleic acids, the use of reduced molecules of the invention in a cosmetic composition and/or a supplemental dietary can contribute to decrease the intracellular ROS level and so to decrease the cellular damages mediated by ROS.

The term “*alcohol*” or “*bioalcohol*” more specifically designates a linear or branched alcohol, diol or triol comprising from 1 and 5 carbon atoms, preferably from 1 to 4 carbon atoms. Specific and preferred examples of “alcohols” include C₁₋₄ alcohols selected from methanol, ethanol, propanol, isopropanol, propanediol, butanol, 2,3-butanediol, 1,4-butanediol, isobutanol, or glycerol, more preferably ethanol.

Polyols are alcohols containing multiple hydroxyl groups. The sugar alcohols are useful compounds in food industry, used for example for increasing the palatability of food or

beverages. In particular, the methods of the invention allow the production of maltitol, sorbitol, xylitol and isomalt. The polymeric polyols can be used in polymer chemistry as reactants to make other polymers. For examples, polymeric polyols can be reacted with isocyanates to make polyurethanes.

- 5 Examples of amino acids include, without limitation, lysine, methionine, threonine, proline, glutamic acid, homoserine, isoleucine, and valine.

Vitamins which can be produced using bacteria or methods of the invention include pantoate, trans-neurosporene, phylloquinone and tocopherols.

- 10 Examples of sterols which can be obtained using bacteria or methods of the invention include steroid compounds with long (8-10 carbons) aliphatic side-chains (usually at position 17) and at least one alcoholic hydroxyl group, usually at position 3. In particular, the methods of the invention allow the production of squalene, cholesterol, testosterone, progesterone and cortisone. Sterols and related compounds play essential roles in the physiology of animals. For example, cholesterol affects the cell membrane's fluidity and
15 serves as secondary messenger in developmental signaling. Corticosteroids, such as cortisol, act as signaling compounds in cellular communication and general metabolism.

- Flavonoids generally refer to ketone-containing compounds. Flavonoids have an anti-oxidant activity similar to the anti-oxidant activity of vitamin C. Flavonoids can also have anti-allergic, anti-inflammatory, anti-cancer, and anti-diarrheal activities. In particular, the
20 methods of the invention allow the production of quercetin and epicatechin. Such flavonoids can be obtained by using recombinant bacteria of the invention.

- The bacteria and methods of the invention can also be used for the production of dicarboxylic acids, and preferentially C5-C30 dicarboxylic acids, having the general formula $\text{HOOC}-(\text{CH}_2)_n-\text{COOH}$. They are particularly suited to produce saturated
25 dicarboxylic acids (e.g., glutaric acid, adipic acid, pimelic acid, suberic acid, azelaic acid, sebacic acid), aromatic dicarboxylic acids (e.g., phthalic acid, isophthalic acid, terephthalic acid) and unsaturated dicarboxylic acids (e.g., glutaronic acid, traumatic acid, muconic acid). Dicarboxylic acids are suitable substrates for preparation of organic acids for the pharmaceutical and food industries. Furthermore, they are useful materials for the

preparation of fragrances, polyamides, adhesives, lubricants, and polyesters. In specific embodiments, dicarboxylic acids can be obtained by using a recombinant bacterium of the invention comprising a recombinant cytochrome P450.

5 In the context of the invention, fatty acids include both saturated and unsaturated fatty acids, with long aliphatic chains (i.e. aliphatic tails longer than 6 carbons). In particular, the methods of the invention allow the production of capric acid, myristic acid, palmitic acid and lignoceric acid (saturated fatty acids) and of palmitoleic acid, oleic acid, linoleic acid and erucic acid (unsaturated fatty acids).

The methods of the invention may also be used to produce lipids, particularly isoprenoids. 10 Indeed, the isoprenoid biosynthesis starts from both the isopentenyl diphosphate (IPP) and the dimethylallyl pyrophosphate (DMAPP). One pathway for synthesizing IPP and DMAPP comprises the production of mevalonate (MEP) from pyruvate. This reaction requires the use of 1-deoxy-D-xylulose-5-phosphate reductoisomerase, which is an NADPH dependent enzyme. Isoprenoids are useful for diverse applications as biofuels, 15 pigments, antioxidants, flavors, or in the pharmaceutical field.

In a particular embodiment, the method of the invention is used to produce carotenoids. Carotenoids have many physiological functions, most of them having an antioxidant activity. Furthermore, the products of degradation of carotenoid (e.g., ionones, damascones and damascenones) are useful material for the preparation of perfumes and more generally 20 in fragrance industry.

In a particular embodiment, the method of the invention is used to produce biofuel. The term biofuel according to the invention comprises "first generation biofuel" and/or "second generation biofuel". First generation biofuels are obtained from vegetal or animal organic material, preferably from sugar, starch, vegetable oil or animal fats. The main source for 25 the production of first generation biofuels are edible plants or parts thereof. The first generation biofuels include vegetal oil, biodiesel, bioalcohols, biogas, syngas and solid biofuels. Bioalcohols include ethanol, propanol and butanol. The second generation biofuels are produced preferably from non-edible plants or non-edible parts of plants. They include non-food crops, biomass wastes, stalks of wheat, corn and wood.

Reactors

In particular embodiments, the methods of the invention are performed in reactors. By “reactor” is meant a conventional tank or any apparatus or system for fermentation and/or bioconversion, typically selected from bioreactors, biofilters, rotary biological contactors, and other gaseous and/or liquid phase bioreactors. The apparatus which can be used according to the invention can be used continuously or in batch loads.

In the reactor, to implement the method of the invention, at least one bacterium or bacterial extract of the invention is used, whilst said reactor is arranged and supplied so that physicochemical conditions are set up and maintained therein so that said bacterium is operational. Depending on the bacterium used, the method may be conducted under aerobiosis, anaerobiosis or microaerobiosis. The process of the invention may thus preferably be performed at a temperature of about 40°C or more, particularly a temperature comprised between 40-70°C, under acid pH conditions, and/or in the presence of ethanol.

Recombinant bacterium

In order to improve their performances or to confer new activities, the bacteria of the invention may be modified to contain one or several recombinant genes. They are particularly suited to contain and express recombinant genes encoding NADPH-dependent enzymes.

In a specific embodiment, a bacterium of the invention comprises a recombinant nucleic acid sequence encoding an NADPH-dependent alcohol dehydrogenase. Such bacterium can be used for producing ethanol, by growing this bacterium in a culture medium comprising for example glucose as carbon source.

For example, strain DG03 as disclosed in the experimental section has been engineered in order to contain an NADPH-dependent alcohol dehydrogenase encoding gene from *Moorella sp* HUC 22-1 into its chromosome (SEQ ID N°3). The expression of this gene confers a particular advantage on the bacterium for the production of bioethanol.

In another specific embodiment, a bacterium of the invention comprises a recombinant Cytochrome P450. Such a bacterium can be used for bioconverting fatty acids into w-

alcohols and corresponding dicarboxylic acids. More precisely, the recombinant Cytochrome P450 catalyzes the required oxidation of the fatty acids for obtaining corresponding dicarboxylic acids. The recombinant bacterium is for example cultivated in a culture medium comprising a carbon source and the fatty acids to be bioconverted.

- 5 For example, a *Deinococcus* of the invention, having a natural and permanent PP/G ratio of at least 0.5, may be engineered in order to contain an NADPH-dependent cytochrome P450 encoding gene from *Bacillus megaterium* (P450BM-3(CYP102A1)) into its chromosome (SEQ ID N°4). The expression of this gene confers a particular advantage on the bacterium for the bioconversion of fatty acids.

10

A recombinant gene or nucleic acid may be inserted into the genome of the bacterium, or inserted as (autonomously) replicating molecules, e.g., on a plasmid, episome, artificial chromosome, etc., as disclosed above. In a preferred embodiment, the recombinant nucleic acid is inserted in the genome of said bacterium in replacement of an endogenous gene.

- 15 Expression of the expressed recombinant genes may be verified using e.g., quantitative PCR, Western blot or by enzymatic assays known per se in the art.

According to an embodiment of the invention, at least one nucleic acid sequence of a bacterium of the invention, encoding a NADPH-dependent enzyme, is used to transform a microorganism, such as a bacterium or a yeast, to provide a recombinant microorganism with a metabolic pathway ratio between Pentose phosphate and glycolysis greater than 0.5.

20 Alternatively or in addition, the bacterium of the invention can be further modified to delete glycolysis genes. For example, a bacterium of the invention has at least one disrupted gene among the phosphoglucose isomerase, the G6P dehydrogenase and both pyruvate kinase isoenzymes. The resulting recombinant bacterium uses further the PP pathway as the primary route of glucose catabolism. For example, a recombinant bacterium having both pyruvate kinase isoenzymes disrupted reroutes its metabolic flux via the combined reactions of phosphoenolpyruvate carboxylase and malic enzymes.

25 In a particular embodiment, the selected gene is deleted, in all or in part, and does not encode a functional protein. The target gene may be inactivated in said bacterium or an

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ancestor thereof, by homologous recombination, gene replacement, or targeted mutagenesis, or any other technique known per se in the art. In a preferred embodiment, the gene is inactivated by deletion of at least part of said gene, which may be replaced by heterologous nucleic acid (e.g., a selection marker). In a preferred embodiment, the bacterium of the present invention lacks a portion of said gene, preferably at least 100 consecutive nucleotides thereof, more preferably at least 200, 300, 400 or 500.

Compositions

A further aspect of the invention resides in the use of bacteria of the invention to make compositions with particular properties, such as antioxidant properties. Such compositions can be cosmetic compositions, nutritional compositions, including dietary supplements and nutraceutical compositions.

For example, a dietary supplement can be prepared, including bacteria of the invention, or extract thereof, and/or compound(s) produced by such bacteria, to supplement the diet and to provide nutriments, such as vitamins, fatty acids or amino acids, that may be missing or may not be consumed in sufficient quantities in a person's diet.

In a particular embodiment, a dietary supplement of the invention contains bacteria producing reduced molecules, or extract thereof. Such dietary supplements can be proposed as pills comprising a bacterial extract powder of bacteria producing reduced molecules, alone or in combination with other components. Otherwise, the pills can contain purified reduced molecules, alone or in combination with other components.

In the context of the invention, cosmetic compositions, or beauty products, relate to compositions suitable for application on at least a part of the body, for cosmetic effects. Cosmetic compositions of the invention include lotions, such as hair lotion and aftershave lotion, skin creams, such as day cream, anti-wrinkle cream and moisturizing cream, or make-up, such as lipstick, etc.

For example, bacteria (or extract thereof) producing reduced molecules are used as an active ingredient in an amount effective to act as an antioxidant agent. Cosmetic compositions of the invention can contain in addition or instead of bacteria, purified reduced molecules.

Preferentially, the composition further comprises one or more cosmetically acceptable carriers or diluents.

Co-cultures

5 A further aspect of the invention resides in microorganism co-cultures having improved properties. More specifically, the invention relates to co-cultures using *Deinococcus* bacteria of the invention, which co-cultures have improved NADPH-dependent enzymatic activities.

10 In a particular embodiment, the invention relates to a co-culture of at least two distinct microorganisms, wherein at least one of said microorganisms is a *Deinococcus* bacterium presenting a metabolic pathway ratio between Pentose phosphate and glycolysis greater than 0.5, and preferably greater than 1, and at least one of said microorganisms is a prokaryotic or eukaryotic cell, and wherein said at least two microorganisms are symbiotic to each other. The prokaryotic or eukaryotic cell may be selected, e.g., from bacteria, yeasts, plant cells, fungi, and mammalian cells. Examples of yeasts include, without
15 limitation, *Saccharomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Pichia*, etc. Examples of bacteria include *Deinococcus* bacteria, *Bacillus sp.*, *E. Coli*, *Clostridium sp.*, etc.

Two microorganisms are considered symbiotic to each other when both require the other for its survival and growth.

20 Co-cultures of the invention may comprise more than 2 distinct microorganisms, such as 3 or 4.

Also, co-cultures may be simultaneous or sequential, preferably simultaneous.

In this regard, a specific object of the invention is a culture of at least two distinct microorganisms, wherein at least one of said microorganisms is a *Deinococcus* bacterium presenting a metabolic pathway ratio between Pentose phosphate and glycolysis greater
25 than 0,5, and at least one of said microorganisms is a yeast.

These co-cultures offer improved range of enzymatic activities and represent valuable products for industrial processes.

Further aspects and advantages of the invention will be disclosed in the following examples, which should be considered as illustrative and do not limit the scope of this application.

5 EXAMPLES

A] Identification and characterization of bacteria having a PP/G ratio superior to 0.5

Stress resistant bacteria are isolated from an environmental sample performing an UV treatment of 4 mJ/cm² for 30 seconds, repeated five times at an interval of 4 hours.

10 The remaining living bacteria are selected. Among these, two *Deinococcus geothermalis* strains (DG01 and DG02) are identified as presenting a PP/G ratio of interest. More particularly, the DG01 strain presents a PP/G ratio of 4.26, and the DG02 strain presents a PP/G ratio of 1.27.

In the following, a method of measurement of the metabolic pathway ratio for these two
15 isolated strains is presented. This method consists on a ¹³C metabolic flux analysis as described in *Krömer et al.*

Cultivations in the parallel bioreactor system for flux analyses

Chemostat cultivations were performed for *Deinococcus geothermalis* strains DG01 and DG02 in a 15-vessels reactor system. Medium n°6 containing 10g/L glucose was used both
20 in the batch phase and in the chemostat phase. The medium was supplemented with 10-fold vitamin solution (vitamin content in the medium 10 µg/L). Cultivation volume was 200ml. Agitation rate was 800 rpm and set point for pH was 7.0 1M NaOH was used to control pH. All cultivations were aerated with ambient air. Three levels of flow rates were used: triplicate cultivations with both strains using 10 ml/min aeration, duplicate
25 cultivations with both strains using 30 ml/min aeration and duplicate cultivation with both strains using 65 ml/min aeration. One vessel was not inoculated but was kept as a control.

Chemostat feed was started 24 hours after the inoculation. Feed rate was 6.0 ml/h for each vessel. This corresponds to a dilution rate of 0.03/h. Chemostat feed was changed to ¹³C-labeled feed after 283 hours (1 g/L universally ¹³C-labeled glucose, 9 g/L natural glucose).
30 Samples of 150 mL were taken after 49 hours of starting the labeled glucose feed. Samples

were centrifuged and cell pellets were frozen at -20°C. After harvesting the cells, chemostat cultivations were continued with the medium containing natural glucose.

Sample preparation and NMR spectroscopy for ¹³C metabolic flux ratio analysis

Labeled cells, harvested from the chemostat cultivations above, were hydrolyzed with 6M
5 HCl at 110°C for 20 hours. In the hydrolysis cells are broken down and the proteins are
hydrolyzed to free amino acids. 16 of the 20 proteinogenic amino acids remain intact; Asn,
Gln, Cys and Trp are destroyed. Acid was removed by a rotary evaporator, washed with
water and freeze dried. For NMR analysis the evaporated samples were dissolved in 500 µl
10 of D₂O. Due to residual HCl, pH remains below 1, as spectrometer equipped with CPQCI
cryoprobe. Two HSQC spectra from each sample were recorded corresponding to the
aliphatic and aromatic areas of the spectrum.

ReTrace pathfinding for the metabolic model

The reconstruction of pathways of central carbon metabolism and amino acid biosynthetic
pathways from their precursors was performed with ReTrace. ReTrace is a recent
15 computational pathway analysis method (Pitkänen E, Jouhten P, Rousu J: Inferring
branching pathways in genome-scale metabolic networks with ReTrace). In press, *BMC
Syst. Biol.*, 2009, which can be queried to discover branching metabolic pathways in a
universal metabolic database. Specifically, ReTrace aims to find pathways which transfer
as many atoms from source to target metabolites as possible. Atom mappings, that describe
20 how atoms are transferred in a reaction from substrate to product metabolites, are defined
in the RPAIR database, which is a sub -database of KEGG.

SwissProt database was queried with *Deinococcus* orf's by blastp (Altschul SF, Madden
TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ : Gapped BLAST and PSI-
BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997,
25 25:3389-3402) with the following options:

```
Blastp -outfmt 6 -query <input file name> -db swiss -out <output file name>
```

Each reaction in the KEGG database was assigned a score by taking the maximum BLAST
score all SwissProt-*Deinococcus* strain sequence pairs, where SwissProt sequence had

been annotated with an EC number corresponding to the reaction. Reaction scores reflected the degree of evidence from the detection of sequence homology that there exists an enzyme catalyzing the reaction in the strains of *Deinococcus*.

ReTrace was run with default settings with the reactions scores from the genome evidence.

- 5 All reactions were considered bidirectional. To capture the paths in central carbon metabolism and in amino acid synthesis, ReTrace was run for several substrate and product metabolites.

Constructing the metabolic models

- 10 Stoichiometric metabolic models of central carbon metabolism were manually set up for *Deinococcus* strains DG01 and DG02 from the path finding results. To generate gapless models some reactions were included with low or no support from the genome data. Prokaryotic genome-wide metabolic models of *Escherichia coli* and *Bacillus subtilis* were examined for modeling the electron transfer chain, oxidative phosphorylation and transport reactions since the path finding does not support searching them. The stoichiometric
15 models were augmented for example with annotation and scoring information.

Metabolic flux ratio analysis

- The software FCAL (R.W. Glaser; FCAL 2.3.1. Szyperski T, Glaser RW, Hochuli M, Fiaux J, Sauer U, Bailey JE, Wüthrick K : Bioreaction network topology and metabolic flux ratio analysis by biosynthetic fractional ¹³C labeling and two-dimensional NMR spectroscopy. *Metab. Eng.* 1999, 1: 189-197.) was used for the integration of ¹³C-scalar
20 fine structures of proteinogenic aliphatic and aromatic amino acid carbon signals in the ¹H-¹³C-HSQC NMR spectra of the sample containing the strain DG01 and the sample containing the strain DG02, and for the calculation of relative abundances of intact carbon fragments originating from a single source molecule of glucose.

- 25 The nomenclature used for the intact carbon fragments, fragmentomers, has been described by Szyperski (Biosynthetically directed fractional ¹³C-labelling of proteinogenic amino acids. An efficient tool to investigate intermediary metabolism. *Eur. J. Biochem.* 1995, 232: 433-448).

Fragmentomer information obtained from proteinogenic amino acids can be traced back to the metabolic intermediates in central carbon metabolism since the carbon backbones are conserved in the amino acid synthesis pathways. Mass balance equations of the specific carbon fragments of the metabolic intermediates were formulated from the propagated
5 fragmentomer information for junctions in central carbon metabolism.

The upper bound for the fraction of phosphoenolpyruvate (Pep) from the pentose phosphate pathway (PPP) was calculated as described by Maaheimo and co-workers (Maaheimo H, Fiaux J, Gakar PZ, Bailey JE, Sauer U, Szyperski T: Central carbon metabolism of *Saccharomyces cerevisiae* explored by biosynthetic fractional ¹³C labeling
10 of common amino acids. *Eur. J. Biochem.* 2001, 268: 2464-2479). The fraction of Pep originating from phosphoenolpyruvate carboxykinase activity, denoted by X_{PEPck} , was calculated from the ratio of the fraction of Pep molecules containing an intact C1 –C2 fragment and a cleaved bond between C2 and C3 and the fraction of oxaloacetate (Oaa) molecules containing the equivalent fragments. Phosphoenolpyruvate carboxykinase is
15 assumed to be the only source of such molecules. Phe-C α , Tyr-C α and Asp-C α , Thr-C α can be traced back to the C2 of Pep and Oaa, respectively.

The contribution of the anaplerotic interconversion of Pep into Oaa, which is participating in the TCA cycle, was also calculated. In calculation of anaplerotic replenishment of TCA cycle by synthesis of Oaa from Pep via the joint action of pyruvate kinase and pyruvate
20 carboxylase the condensation of AcCoA and Oaa is assumed irreversible. No hit for citrate lyase, catalysing the reaction in the opposite direction, was found in the genome evidence –supported path finding. The Oaa molecules originating from oxoglutarate (Oga) through the TCA cycle possess cleaved C2-C3 bonds, allowing the measurement of the fraction of Oaa originating from anaplerotic carboxylation and the fraction of intact C2-C3
25 connectivities in Oaa. The intact fragments in Oaa were obtained from the intact fragments in Oga since the C2-C3-C4 fragment of Oaa is conserved in the TCA cycle in synthesis of Oga. The intact fragments in biosynthetic precursor Oga were deduced from Glu.

The upper and lower bounds for pyruvate (Pyr) originating from the malic enzyme reaction, denoted by $X_{\text{MAE}_{ub}}$ and $X_{\text{MAE}_{lb}}$ respectively, were calculated from a mass
30 balance of intact C2-C3 fragments of Pyr. The upper and lower bounds were obtained

from the assumption that the substrate fragment for malic enzyme has an equally conserved connectivity as Oga and Oaa

The skilled person can easily implement the method described above to identify and characterize further bacteria having a PP/G pathway ratio superior or equal to 0.5, from
5 any sample comprising bacteria.

B] Production of deinoxanthine

The deinoxanthine is a specific orange-red pigment, close to astaxanthin. Deinoxanthine is well known to be a powerful antioxidant, immuno-stimulants and photo-protective compound.

10 In the following, the production of deinoxanthine, using the method of bioproduction of the invention with an isolated *Deinococcus radiodurans* (DR04), is described. The bacterium DR04 is identified and isolated according to the method described above. This bacterium presents a PP/G ratio superior to 0.5.

Material and method:

15 Complex Medium Glucose contains: peptone 2g/L, yeast extract (Lot BCBD0078V; Ref: 70161 Fluka) 5g/L and glucose (Lot 080M0143V; Ref : 68270 SIGMA) 10g/L in osmosed water: solution sterilized by autoclaving (15 minutes at 120°C). To this solution are added the following solutions: MOPS buffer solution (10X) pH7 [acid MOPS 400mM , NH₄Cl 200mM , NaOH 100mM , KOH 100mM , CaCl₂ 5μM, Na₂SO₄ 2.76mM, MgCl₂ 5.28 mM];
20 micronutrients (10000X) [(NH₄)₆(Mo₇)₂₄ 300mM, H₃BO₃ 4mM, CoCl₂ 0.3mM, CuSO₄ 0.1mM, MnCl₂ 2.5 mM, ZnSO₄ 0.1mM]; vitamins (10000X) [10 mg/L of each: D-biotin, Niacin (nicotinic acid), Pyridoxin (pyroxidal hydrochloride) B6, Thiamin (vitamin B1 hydrochloride) – Stock pH4 – filter sterilized (0.22 μm)]; FeCl₃(100X) 2mM in C₆H₅Na₃O₇ 20mM filter sterilized (0.22 μm); K₂HPO₄ 100 g/L, autoclaved.

25 All solvents for high performance liquid chromatography (HPLC) were super purity solvents and purchased from Sigma Aldrich. 2,6-Di-r-butyl-p-cresol (BHT) in purum quality and NaB& in purum p.a. quality were purchased from Sigma Aldrich.

Bacteria and culture procedure

Deinococcus sp were inoculated on Complex Medium Glucose. The bacteria were grown under aerobic conditions and continuous shaking at 45°C or 30°C during seven days. The culture reached its stationary phase and bacteria could be harvested by centrifugation. The pellet average weight was 5 g per liter of culture.

5 Extraction and purification

A bacteria cell pellet of 500 mg was washed two times with 50 mL of ultra pure H₂O. Then the washed bacteria cell pellet was mixed with 1 ml of Methanol containing 1% of BHT as antioxidant under argon, homogenized for 10 minutes and then centrifuged. The pellet was reextracted three times until it turned totally colorless. The extracts were
10 combined.

LCMS studies

The crude material was purified by Hypersil ODS (4.6x250mm, 5µm) column, on a Dionex HPLC system using a mix of acetonitrile/methanol/isopropanol (40/50/10) (v/v) as mobile phase, a flow rate of 0.8 ml/min, and the detector set at 480 nm.

15 HPLC assay of deinoxanthine in an extract of DR04 was used to assess the amount of deinoxanthine produced by the bacterium. It can be expressed as mg / g of dry biomass.

Results

The amount of deinoxanthine contained in the extract of DR04 was 0.1mg/g dry biomass.

Such extract can be used advantageously to prepare a cosmetic composition and/or a
20 supplemental dietary, with antioxidant properties.

C] Production of carotenoids

Carotenoids are organic pigments, produced by some bacteria to protect themselves from oxidative attack. Carotenoids, well known to be powerful antioxidant compounds, cannot
25 be manufactured by mammal animals, so that they obtain carotenoids in their diets. Mammal animals employ carotenoids in various ways in metabolism.

In the following, the production of carotenoids, using the method of bioproduction of the invention with an isolated *Deinococcus geothermalis* (MX6-1E_14), is described. More precisely, carotenoids are produced using *Deinococcus* cells cultivated on culture medium containing either glucose or xylose as sole carbon source. The *Deinococcus* cells have been
5 previously identified and isolated according to the method described above. These bacteria present a PP/G ratio superior to 0.5.

Material and methods:

Medium N°6 composition:

Reducing sugar (20 g/L) ; MOPS acid 40 mM ; NH₄Cl 30 mM ; NaOH 10 mM ; KOH 10
10 mM ; CaCl₂.2H₂O 2 μM ; Na₂SO₄.10H₂O 0,1 mM ; MgCl₂.6H₂O 0,1 mM ;
(NH₄)₆(Mo₇)O₂₄.4H₂O 10 nM ; H₃BO₃ 100 nM ; CoCl₂.6H₂O 10 nM ; CuSO₄.5H₂O 200
nM ; MnCl₂ 1 μM ; ZnSO₄.7H₂O 100 nM ; D-Biotin 10 μg/L ; Niacin (nicotinic acid) 10
μg/L ; Pyridoxin (pyridoxal HCl ou vitamine B6) 10 μg/L ; Thiamin HCl (vitamine
B1) 10 μg/L ; vitamin B12 10 μg/L ; FeCl₃ 200 μM ; Sodium Citrate.2H₂O 200 μM ;
15 K₂HPO₄ 0,5 g/L.

Bacteria and culture procedure:

Deinococcus strain MX6-1E_14 strain was cultivated in 500 ml medium N°6 containing
either 20g/l of glucose or 20g/l of xylose in 1L fermentor at 45°C under 0,1 L/min of
20 aeration.

Extraction and purification:

Carotenoids extraction was performed as follow:

- the bacteria were harvested at stationary phase and centrifuged at 400 rpm ;
- 25 - 10 mg of pellets were taken in 1 ml of methanol, and vortexed 3 min ;
- the sample was then centrifuged 3 min at 10000 rpm at 4°C ;

- the supernatant was recovered and absorbance measured at 540 nm to quantify the carotenoids (the amount of carotenoids is proportional to the absorbance at 540 nm).

5 Results

In the *Deinococcus* strain used in the experiment, about 50% of glucose is metabolized by glycolysis and 50% by pentose phosphate pathway. This bacterium generates a ratio NADH/NADPH of 1, that is optimal for the production of carotenoids.

Extraction from glucose culture medium: $OD_{540nm} = 0.042$

10 Extraction from xylose culture medium: $OD_{540nm} = 0.012$

In the xylose culture medium the NADH/NADPH ratio is modified in favor of NADH (i.e., xylose generates only NADH), and the production of carotenoids is decreased compared to the production of carotenoids using the same strain in the glucose culture medium, wherein the NADH/NADPH ratio remains equal to 1.

15

D] Recombinant bacterium: Insertion of an NADP-dependent ADH encoding gene

The metabolic flux ratio analysis above shows that in DG02 strain up to 56% of glucose is consumed through the Pentose Phosphate Pathway, indicating that an important part of NADPH is produced as redox potential. In order to use this NADPH pool for ethanol
20 production, an NADP-dependent ADH encoding gene from *Moorella sp* HUC 22-1 (SEQ ID N°3) is introduced into the chromosome of strains DG02.

Materials and methods

Bacterial strains and growth conditions:

Escherichia coli (*E. coli*) strain SCS110 is used to propagate plasmids. The bacteria are cultivated at 37°C and 200 RPM in Luria-Bertani (LB) Broth (per liter : Tryptone 10g, Yeast extract 5g, Sodium chloride 10g). Solid media is prepared by addition of Agar 1.5%.

Deinococcus bacteria DG02 are cultivated at 45°C and 200RPM in PGY. The composition of the PGY medium is the following, per liter: Peptone (10g), Yeast extract (5g) and Glucose (1g). Composition of the solid media is, per liter: Peptone (10g), Yeast extract (5g), Glucose (1g) and Agar (15g).

Transformation:

E. coli transformation is done using commercial competent cells SCS110 from Stratagene or JM109 from Promega.

For *Deinococcus* cells, a fresh culture in stationary phase is diluted 100 times in 50 ml of PGY. Cells are grown until late exponential phase ($OD_{600nm} = 0.8$); the pellet is resuspended in an appropriate volume of ice cold 2xPGY/10%v/v Glycerol/30mM $CaCl_2$. For transformation, desired amount of plasmid DNA is added to 100 μ l of the cells. The mixture is incubated 30 minutes on ice transferred at 42°C for 90 seconds and back to ice for 5 minutes. 200 μ l of fresh 2XPGY medium is added and the transformants are shaken at 200RPM and 37°C during 2 hours. They are serially diluted and spread on appropriate selective PGY plates.

DNA manipulation:

Plasmid miniprep from *E. coli* cells is done using the QIAGEN minipreps DNA purification system and midiprep is done using the Plasmid DNA purification NucleoBond® Xtra Midi Plus EF kit from Macherey-Nagel. These preparations are done from 3-100 ml of *E. coli* culture in stationary phase.

The oligonucleotides are synthesized by Eurogentec. The polymerases used for PCR amplification are the PHUSION Hi-Fidelity polymerase from Finzyme; and the KOD Xtreme-hot start DNA polymerase from Novagen for overlapping PCRs. PCR fragments are cleaned up using the Wizard SV Gel and PCR Clean-Up System kit from Promega.

Genetic materials are separated by agarose gel electrophoresis. DNA is quantified with a Biophotometer from Eppendorf.

DNA inserts are synthesized by Genecust Europe and cloned into appropriate vector.

Method of genetic insertion into Deinococcus chromosome:

5 Insertion of DNA fragments into the chromosome of *DG02* is performed using homologous recombination mechanism. The cassette of insertion contains an operon with the alcohol dehydrogenase encoding gene (*adh_{zm}*) from *Z. mobilis*, placed under the control of the *pgroESL* promoter and, a *pTufA-bleo* bleocin resistance cassette. The DNA sequence of the region that has to be inserted is flanked by 500 bp regions homologous to
10 the sequences upstream or downstream the chromosomal target. The insertion cassette is carried by pMD66 thermosensitive shuttle vector, transformed into *Deinococcus* and high temperature exposure (52°C for 4 days) is used to allow for chromosomal insertion and plasmid loss.

The transformants that have incorporated the region of interest into the chromosome
15 (named DG03) are selected on PGY medium containing bleocin.

Correct insertions are checked by PCR on genomic DNA, and sequencing of the modified chromosome on the region of interest.

Alcohol dehydrogenase activity test:

4 ml of pararosaniline (Sigma) at 2.5 mg/ml in absolute ethanol are added to 200 ml of LB
20 agar containing 50 mg of sodium bisulfite (Conway et al, 1987b). 2-days-old *DG03* cells grown on TGY agar plates (supplemented if necessary with bleocin) are plated on the indicator plates and incubated at 37°C for 2 to 3 hours.

Ethanol production:

25 The ability of DG03 to produce ethanol from whole wheat 3%- or 6%-containing medium, in aerobic conditions is evaluated. The test is carried out at 30°C.

From pre-cultures (in stationary phase) prepared in Complex medium Glucose [peptone 2g/L, yeast extract 5g/L and glucose 10g/L in osmosed water: solution sterilized by autoclaving (15 minutes at 120°C). To this solution are added the following solutions: MOPS buffer solution (10X) pH7 [acid MOPS 400mM, NH₄Cl 200mM, NaOH 100mM, 5 KOH 100mM, CaCl₂ 5μM, Na₂SO₄ 2.76mM, MgCl₂ 5.28 mM]; micronutrients (10000X) [(NH₄)₆(Mo₇)₂₄ 300mM, H₃BO₃ 4mM, CoCl₂ 0.3mM, CuSO₄ 0.1mM, MnCl₂ 2.5 mM, ZnSO₄ 0.1mM]; FeCl₃(100X) 2mM in C₆H₅Na₃O₇ 20mM; K₂HPO₄ 1g/L: solutions sterilized by filtration (0.2μm)], 6ml of enriched medium [carbon source 10g/L in osmosed water: solution sterilized by autoclaving (15 minutes at 120°C)] are seeded 10 (seeding at 1% v/v).

Bleocin (3 μg/mL the culture medium) is added to this culture medium before the seeding.

Gas Chromatography FID analysis (Varian CP-WAX 57 CB 25m*0.32mm column) is used to quantify ethanol. Residual glucose is quantified by HPLC coupled with refractometry (Phenomenex LUNA 3μm NH₂ 100A 150*4.6mm column, acetonitrile/H₂O 15 85:15 mobile phase).

Ethanol production is monitored after 7 days of growth in whole wheat 3%- or 6%-containing medium, in aerobic conditions (i.e. cultures are left in an incubator, at 30°C, under agitation, for 7 days. The cultures are then centrifuged for 10 minutes at 4000rpm. Supernatants are filtered (0.2μm), poured into other tubes, and placed at -80°C).

20 The expression of the NADP-dependent ADII (SEQ ID N°5) does maximize ethanol production by the DG03 bacteria.

SEQUENCE LISTING IN ELECTRONIC FORM

25 In accordance with section 111(1) of the *Patent Rules*, this description contains a sequence listing in electronic form in ASCII text format (file: 87513-85 SEQ 05-JUN-14 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

30

CLAIMS

1. A *Deinococcus* bacterium having a metabolic pathway ratio between Pentose phosphate and glycolysis greater than 0.5 and equal or lower than 5.
2. The bacterium of claim 1, which is obtained by a method comprising the following steps:
 - a) providing a sample comprising bacteria;
 - b) subjecting the sample to a cell-destructing DNA damaging treatment;
 - c) identifying or isolating living or growing bacteria from said treated sample;
 - d) measuring the metabolic pathway ratio between Pentose phosphate and glycolysis of bacteria of step c); and
 - e) selecting a *Deinococcus* bacterium of step d) which has a metabolic pathway ratio Pentose phosphate/glycolysis superior to 0.5 and equal or lower than 5.
3. The bacterium of any one of claims 1 and 2, wherein said bacterium can utilize lignin, cellulose, hemi-cellulose, starch, xylan, polysaccharide, xylose, galactose, sucrose, lactose, glycerol, molasses or glucose, as carbon source.
4. The bacterium of any one of claims 1 to 3, further comprising at least one recombinant nucleic acid molecule encoding an NADPH-dependent enzyme.
5. The recombinant bacterium of claim 4, wherein the at least one recombinant nucleic acid molecule encodes an NADPH-dependent Alcohol dehydrogenase and/or an NADPH-dependent cytochrome P450.
6. Use of the bacterium of any one of claims 1 to 5, for the bioproduction of a compound of interest.
7. The use of claim 6, wherein the compound is a metabolite selected from alcohols, acids, amino acids, vitamins, sterols, flavonoids, fatty acids, isoprenoids, and polyols.

8. The use of claim 6, wherein the compound is a pharmaceutical compound or nutrient selected from a protein, polypeptide, peptide, chemical compound, lipid, or saccharide, having a biological activity.
9. Use of the bacterium of any one of claims 1 to 5, for the bioconversion of a substrate into a substance of interest.
10. The use of claim 9, for the bioconversion of aliphatic compounds into dicarboxylic acids.
11. Use of the bacterium of any one of claims 1 to 5, for the production of an anti-oxidant or reduced molecule.
12. The use of claim 11, wherein the antioxidant molecule is selected from glutathione, deinoxanthine, vitamin C or E, and antioxidant enzymes.
13. Use of the bacterium of any one of claims 1 to 5, for the preparation of a dietary supplement and/or for the preparation of a cosmetic composition.
14. A method for the production of a compound of interest, the method comprising selecting the bacterium of any one of claims 1 to 5, which produces said compound, culturing said bacterium in an appropriate culture medium comprising a source of carbon, and recovering the compound of interest from the bacterium or culture medium.
15. A method for the bioconversion of a substrate into a substance of interest, the method comprising selecting the bacterium of any one of claims 1 to 5 having said bioconversion activity, culturing said bacterium in an appropriate culture medium comprising a source of carbon and the substrate(s), and recovering the substance of interest from the bacterium or culture medium.
16. The method of claim 15, for the bioconversion of aliphatic compounds into dicarboxylic acids.

17. A method for the production of a reduced molecule, the method comprising selecting the bacterium of any one of claims 1 to 5, which produce said reduced molecule in an appropriate culture medium comprising a source of carbon, culturing said bacterium, and recovering the reduced molecule of interest from the bacterium or culture medium.

18. A dietary supplement comprising the bacterium according to any one of claims 1 to 5.

19. A cosmetic composition comprising the bacterium according to any one of claims 1 to 5 and one or more cosmetically acceptable carriers or diluents.

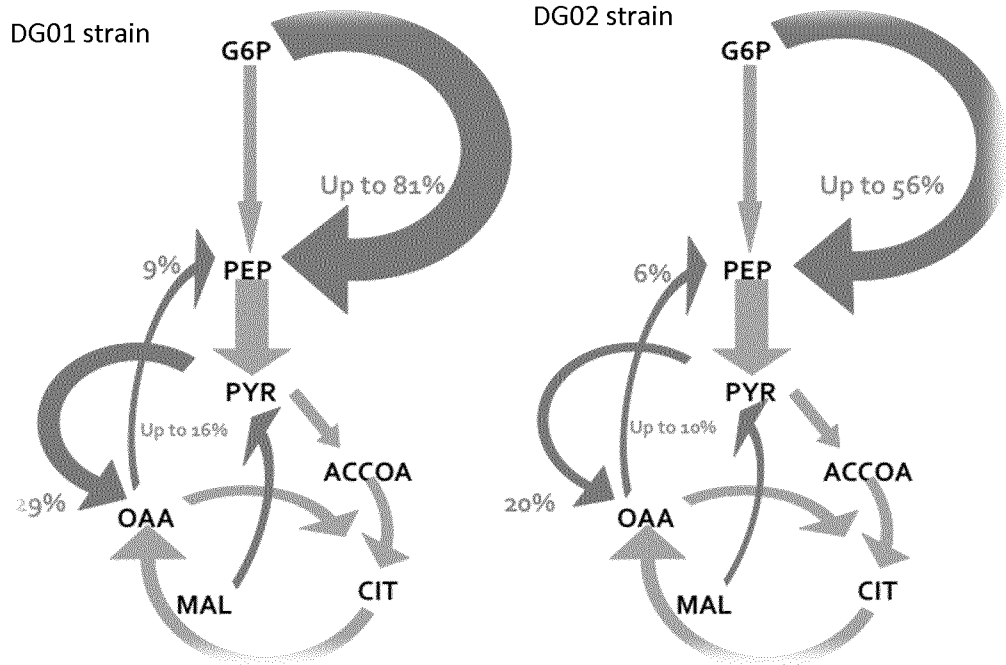


FIGURE 2