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(54) Title: MICROORGANISM EXPRESSING ALDOSE-1-EPIMERASE

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A transformed microorganism capable of converting an aldopentose to a ketopentose at a higher rate than the equivalent microorganism prior to transformation.

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MICROORGANISM EXPRESSING ALDOSE-1-EPIMERASE

FIELD OF THE INVENTION

The present invention relates to a microorganism.

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In particular, the present invention relates to a transformed microorganism capable of: (i) converting an aldopentose to a ketopentose at a higher rate than the equivalent microorganism prior to transformation; and/or (ii) a higher growth rate in the presence of aldopentose than the equivalent microorganism prior to transformation; and/or (iii) a higher metabolism of aldopentose than the equivalent microorganism prior to transformation.

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The present invention further relates to methods for preparing transformed microorganisms capable of: (i) producing a pentose derived compound; and/or (ii) converting an aldopentose to a ketopentose at a higher rate than the equivalent microorganism prior to transformation; and/or (iii) a higher growth rate in the presence of aldopentose than the equivalent microorganism prior to transformation; and/or (iv) a higher metabolism of aldopentose than the equivalent microorganism prior to transformation; said methods comprising the step of transforming a microorganism with a nucleotide sequence encoding an aldose-1-epimerase wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose.

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Further, the present invention relates to inoculums (inocula) and culture media comprising the microorganism according to the present invention or a microorganism prepared by a method according to the present invention.

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The present invention additionally relates to methods for producing biofuel and/or a pentose derived compound comprising culturing a microorganism according to the present invention or a microorganism prepared by a method according to the present invention.

In addition, the present invention relates to a biofuel and/or a pentose derived compound obtained by a method of the present invention.

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Further, the present invention relates to the use of a microorganism according to the present invention or prepared by a method of the present invention for the production of a ketopentose and/or a biofuel and/or a pentose derived compound.

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BACKGROUND

Biofuels are being developed as a green and sustainable alternative to fossil fuels for transportation, heating and energy supply. Rising oil prices have made the production of biofuels more economically feasible, and ultimately the availability of fossil fuels is limited. Bioethanol, a biofuel, is generally considered as being much more CO₂ neutral in comparison with petroleum based transportation fuel. In addition, it is possible to use bioethanol as a partial as well as full gasoline substitute without drastic changes to the engine technology.

Typically, bioethanol is produced by fermentation of sugars derived from agricultural feedstock - such as sugar cane, sugar beet, maize and cereals (such as wheat and corn) - which are starch-rich and sugar-rich plant materials (the remainder of these plant materials is referred to as agricultural waste). However, a problem associated with processes which use these materials is that they utilise what could otherwise have been used for foodstuffs for humans and animal feeds. A consequence of this is that there is a reduction in the amount of foodstuffs and animal feeds which are available which, in turn, increases the price of food.

In fact, it has been predicted that even if the entire maize crop of the USA was used for ethanol production it would not be possible to meet the future demand in the USA. For example, in Spring 2008 the US Department for Agriculture estimated that, based on the amount of US land sown with maize, about 12 billion bushels of maize would be harvested in USA in 2008. Using techniques currently available in the art, 2.8 gallons of ethanol is the average production from 1 bushel of maize. Thus, if the entire 2008 USA maize harvest was made into ethanol, 33 billion gallons of ethanol would be obtained. According to the US Energy Information Administration statistics, however, 142 billion gallons of gasoline was used as fuel for cars and trucks in 2007 in USA. Assuming that there is no significant decline in the demand for gasoline in the USA in 2008, then the supply of the gasoline substitute ethanol from maize could not meet the demand.

Thus, the availability of sources of starch-rich and sugar-rich plant materials is a rate-limiting factor for the production of biofuels.

The so-called agricultural waste material of, for example, sugar cane, sugar beet, sorghum, Soya beans, maize, and cereals (such as wheat and corn) comprises mainly lignocellulosic material. Lignocellulosic material primarily comprises long sugar chains. In general, about two thirds of the sugars of these long chain sugars are hexose sugars (in particular glucose), which are mainly

in the form of cellulose, and about one third of the sugars of these long chain sugars are pentose sugars (in particular xylose and arabinose) present mainly in the form of arabinoxylan polymers. After hydrolysis of cellulose, the hexose sugars can be fermented by the traditional yeast based method. However, cellulose is a robust structure which is very resistant to extraction and enzymatic hydrolysis. Arabinoxylans are comparatively easier to extract and hydrolyse to release, in the main, pentose sugars; but the released sugars can not be fermented into ethanol in sufficiently high concentration by known microorganisms.

Two obstacles for efficient ethanol production from waste plant material are the difficulties associated with depolymerisation of cellulose and the lack of suitable organisms that can metabolize, for large-scale production, pentose sugars into ethanol.

Theoretically, one way to obtain such a suitable organism is to transfer the ability to metabolize pentoses from natural pentose metabolizing organisms into known highly efficient ethanol producers. This has been the subject of much work in various research groups during the last 15-20 years. But with pentoses it has not been possible to obtain metabolic rates comparable to the rates obtained when using glucose. To increase this low metabolic rate has been and is still a subject of discussion and ongoing research and is still a major obstacle for the use of, for example, engineered *S. cerevisiae* in ethanol fermentation from pentoses.

STATEMENTS OF THE INVENTION

Surprisingly, we have found that by modifying microorganisms to express and/or overexpress an aldose-1-epimerase, a faster conversion of aldopentoses into ketopentoses is facilitated (when compared to the equivalent microorganisms prior to modification). In that way, microorganisms can be obtained which are capable of faster conversion of pentose sugars (preferably aldopentose sugars) into a biofuel (preferably a biofuel comprising ethanol) when compared to the equivalent microorganisms prior to modification, which is particularly advantageous for large-scale industrial biofuel production.

Xylulose-5-phosphate is an intermediate (which may be derived from D-xylose, L-arabinose or even D-lyxose), that enters into the pentose phosphate pathway and is further metabolized into ethanol under anaerobic conditions. An entire metabolic pathway from xylose into ethanol is shown in Figure 4.

Ribulose-5-phosphate is an intermediate (which may be derived from D-ribose), that enters into the pentose phosphate pathway and is further metabolized into ethanol under anaerobic conditions.

- 5 In one aspect, the present invention provides a transformed microorganism capable of converting an aldopentose to a ketopentose at a higher rate than the equivalent microorganism prior to transformation.

10 In another aspect, the present invention provides a transformed microorganism capable of a higher growth rate in the presence of aldopentose than the equivalent microorganism prior to transformation.

The present invention additionally provides a transformed microorganism capable of a higher metabolism of aldopentose than the equivalent microorganism prior to transformation.

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The present invention further provides a microorganism wherein said microorganism comprises a nucleotide sequence encoding an exogenous aldose-1-epimerase.

20 In another aspect, the present invention provides a microorganism wherein said microorganism is capable of expressing an exogenous aldose-1-epimerase.

In a further aspect, the present invention provides a microorganism wherein said microorganism comprises a nucleotide sequence encoding an exogenous aldose-1-epimerase, and wherein said microorganism is capable of expressing said exogenous aldose-1-epimerase; and wherein said
25 microorganism is capable of converting an aldopentose to a ketopentose.

Further, the present invention provides a microorganism comprising an expression vector encoding an aldose-1-epimerase, wherein said microorganism is capable of converting an aldopentose to a ketopentose.

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In another aspect, the present invention provides a transformed microorganism wherein said microorganism is capable of expressing an aldose-1-epimerase and wherein said microorganism is capable of converting an aldopentose to a ketopentose.

The present invention provides, in a further aspect, a method for preparing a transformed microorganism capable of producing a ketopentose, said method comprising the step of transforming a microorganism with a nucleotide sequence encoding an aldose-1-epimerase, and wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose.

The present invention provides, in a further aspect, a method for preparing a transformed microorganism capable of producing a pentose derived compound, said method comprising the step of transforming a microorganism with a nucleotide sequence encoding an aldose-1-epimerase, and wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose.

The present invention provides, in a further aspect, a method for preparing a transformed microorganism capable of producing a pentose derived compound at a higher rate than the equivalent microorganism prior to transformation, said method comprising the step of transforming a microorganism with a nucleotide sequence encoding an aldose-1-epimerase, and wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose.

In another aspect, the present invention provides a method for preparing a transformed microorganism capable of converting an aldopentose to a ketopentose at a higher rate than the equivalent microorganism prior to transformation, said method comprising the step of transforming a microorganism with a nucleotide sequence encoding an aldose-1-epimerase, wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose.

In a further aspect, the present invention provides a method for preparing a transformed microorganism capable of converting an aldopentose to a ketopentose at a higher rate than the equivalent microorganism prior to transformation, said method comprising the step of transforming a microorganism such that the expression of an aldose-1-epimerase is upregulated, wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose.

In addition, the present invention provides a method for preparing a transformed microorganism capable of a higher growth rate in the presence of aldopentose than the equivalent

microorganism prior to transformation, said method comprising the step of transforming a microorganism with a nucleotide sequence encoding an aldose-1-epimerase, wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose.

- 5 In a further aspect, the present invention provides a method for preparing a transformed microorganism capable of a higher growth rate in the presence of aldopentose than the equivalent microorganism prior to transformation, said method comprising the step of transforming a microorganism such that the expression of an aldose-1-epimerase is upregulated, wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose.

The present invention additionally provides a method for preparing a transformed microorganism capable of a higher metabolism of aldopentose than the equivalent microorganism prior to transformation, said method comprising the step of transforming a microorganism with a nucleotide sequence encoding an aldose-1-epimerase, wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose.

In a further aspect, the present invention provides a method for preparing a transformed microorganism capable of a higher metabolism of aldopentose than the equivalent microorganism prior to transformation, said method comprising the step of transforming a microorganism such that the expression of an aldose-1-epimerase is upregulated, wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose.

The present invention provides, in a further aspect, a method for producing a pentose derived compound, wherein said method comprises culturing in a culture medium a microorganism according to the present invention or a microorganism prepared by a method of the present invention.

In a further aspect, the present invention provides a method for producing a pentose derived compound comprising the steps of:

- (a) transforming a microorganism with a nucleotide sequence encoding an aldose-1-epimerase wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose; and
- (b) culturing the transformed microorganism in a culture medium.

The present invention provides, in a further aspect, a method for producing a ketopentose, wherein said method comprises culturing in a culture medium a microorganism according to the present invention or a microorganism prepared by a method of the present invention.

5 In a further aspect, the present invention provides a method for producing a ketopentose comprising the steps of:

(a) transforming a microorganism with a nucleotide sequence encoding an aldose-1-epimerase wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose; and

10 (b) culturing the transformed microorganism in a culture medium.

Ketopentoses mentioned herein may be metabolised further within the cell facilitating the production of a range of products, which include but are not limited to: ethanol, lactic acid, succinic acid, acetic acid, acetaldehyde, itaconic acid, cresol, 3-hydroxypropionic acid, poly-3-
15 hydroxyalkanoates, protocatechuic acid, pyrocatechol, guaiacol, veratrol, vanillin, vanillic acid, vanillyl alcohol, muconic acid, adipic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-methoxybenzoic acid, 4-aminobenzoate, 4-hydroxyaniline, 4-methoxyaniline, quinol, anisole, phenol, anthranilic acid, 3-hydroxyanthranilate, 2,3-dihydroxybenzoic acid, 2-aminophenol, 1,4-cyclohexanedione and aromatic amino acids.

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In another aspect, the present invention provides a method for producing a biofuel, wherein said method comprises the step of culturing in a culture medium a microorganism according to the present invention or a microorganism prepared by a method according to the present invention.

25 In another aspect, the present invention provides a method for preparing a transformed microorganism capable of producing a biofuel at a higher rate in a culture medium than the equivalent microorganism prior to transformation, said method comprising the step of transforming a microorganism with a nucleotide sequence encoding an aldose-1-epimerase, preferably in an expression vector encoding same, wherein said transformed microorganism is
30 capable of converting an aldopentose to a ketopentose.

In another aspect, the present invention provides a method for producing a biofuel comprising the steps of:

- (a) transforming a microorganism with a nucleotide sequence encoding an aldose-1-epimerase, preferably in an expression vector encoding same, wherein said transformed microorganism is capable of converting an aldopentose to a ketopentose; and
- (b) culturing the transformed microorganism in a culture medium comprising aldopentose.

In a further aspect, the present invention provides a method for producing a biofuel wherein said method comprises the step of culturing a microorganism in a culture medium, wherein said microorganism comprises a nucleotide sequence encoding an aldose-1-epimerase and wherein said microorganism is capable of expressing said aldose-1-epimerase; and wherein said microorganism is capable of converting an aldopentose to a ketopentose.

In a further aspect, the present invention provides a biofuel obtained or obtainable by a method according to the present invention.

In another aspect, the present invention provides the use of a microorganism according to the present invention or a microorganism prepared by a method of the present invention for the production of a ketopentose.

In another aspect, the present invention provides the use of a microorganism according to the present invention or a microorganism prepared by a method of the present invention for the production of a pentose derived compound.

Further, the present invention provides the use of a microorganism according to the present invention or a microorganism prepared by a method of the present invention for the production of a biofuel.

In another aspect, the present invention provides the use of a microorganism for the production of a biofuel, wherein said microorganism comprises a nucleotide sequence encoding an aldose-1-epimerase, and wherein said microorganism is capable of expressing said aldose-1-epimerase, and wherein said microorganism is capable of converting an aldopentose to a ketopentose.

In a further aspect, the present invention provides an inoculum comprising a microorganism according to the present invention or a microorganism prepared by a method according to the present invention.

Further, the present invention provides a culture medium comprising a microorganism according to the present invention or a microorganism prepared by a method according to the present invention.

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SOME ADVANTAGES

Advantageously, by using the microorganisms according to the present invention, biofuels (such as ethanol) can be produced.

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A further advantage is that by using the microorganisms according to the present invention, biofuels (such as ethanol) can be efficiently produced using pentose sugars. Without wishing to be bound by theory, the modification of microorganisms to express or overexpress an aldose-1-epimerase allows an increase in the rate of inter-conversion between aldopentose anomers and the intracellular conversion of aldopentoses to ketopentoses – in other words it relieves a rate-limiting constraint – which in turn allows for a faster and more efficient production of ethanol by the pentose phosphate pathway than compared to the equivalent microorganism prior to modification.

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More advantageously, by using the microorganisms according to the present invention, biofuels can be produced from waste materials such as agricultural wastes (including cereal straw – such as wheat straw; sugar beet pulp; sugar cane bagasse; stovers - such as sorghum, Soya beans, maize or corn stovers; and wood chips). With the present invention there is no need (or there is a reduced need) to use materials (such as sugar cane extract, sugar beet extract, sorghum starch, maize starch, wheat starch or corn starch) which could otherwise be used as a food source for humans and/or as an animal feed.

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Most advantageously, the present invention provides microorganisms which are capable of metabolising, for large-scale production (such as industrial production), pentose sugars (such as aldopentose sugars) into a biofuel (such as a biofuel comprising ethanol).

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Advantageously, the microorganisms according to the present invention enable the optimum use of the sugars released by hydrolysis of lignocellulosic material by the fermentation of pentose sugars.

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The present invention enables the production of a biofuel which is more CO₂ neutral in comparison with petroleum based transportation fuel. With the present invention, CO₂ emissions will be low (even lower) when producing the biofuel according to the present invention than compared to the production of a typical petroleum based transportation fuel (fossil fuels).

Surprisingly, the present invention shows that the transformation of microorganisms to express aldose-1-epimerase results in an increased conversion rate of pentose sugars to a biofuel.

10 FIGURES

Figure 1. A schematic representation of the metabolic pathways detailing the metabolism of the two most abundant aldopentoses, D-xylose and L-arabinose. Both aldopentoses are converted into a ketopentose, and further into D-xylulose 5-phosphate. Without wishing to be bound by theory, as indicated on the figure, one type of pathway (aldose reductase type) is found in fungi whereas the other type of pathway (isomerase type) is found in bacteria. In the fungal pathway type, the first enzymes may be called "D-xylose reductase" and "L-arabinose reductase", but often the same enzyme can reduce both D-xylose and L-arabinose and may then serve both pathways and be referred to by the less specific name "aldose reductase".

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Figure 2. Without wishing to be bound by theory, Figure 2 shows a schematic representation of fungal and bacterial type of metabolic pathways of some less abundant pentoses (D- and L-lyxose, D-ribose) detailing the initial metabolism until the entry into the pentose phosphate pathway.

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Figure 3. A schematic representation of the non-oxidative part of the pentose phosphate pathway (PPP).

Figure 4. A schematic representation of the pentose phosphate pathway (PPP). Here, ketopentose xylulose-5-phosphate, which may be derived from D-xylose or L-arabinose, is further metabolized into ethanol under anaerobic conditions. XI is xylose isomerase and XK is xylulokinase. The net input of xylose into the process and the net output of ethanol from the process is shown (nettoprocess).

35 DETAILED DESCRIPTION

As used herein the phrase “a transformed microorganism capable of converting an aldopentose to a ketopentose at a higher rate than the equivalent microorganism prior to transformation” encompasses microorganisms transformed with a nucleotide sequence encoding an aldose-1-epimerase, such as an expression vector comprising said nucleotide sequence, and microorganisms transformed to upregulate the expression of an aldose-1-epimerase (in other words overexpress an aldose-1-epimerase).

In one embodiment the microorganism has been transformed with a nucleotide sequence that causes the microorganism to overexpress an aldose-1-epimerase. For example, a promoter is inserted into the genome of a microorganism which enables the microorganism to overexpress an endogenous nucleotide sequence encoding an aldose-1-epimerase.

In another embodiment the microorganism has been transformed with a nucleotide sequence encoding an aldose-1-epimerase. For example, the microorganism is transformed with an expression vector comprising a nucleotide sequence encoding an aldose-1-epimerase operably linked to a regulatory sequence.

Preferably the nucleotide sequence encoding an aldose-1-epimerase mentioned herein is in an expression vector encoding same.

Preferably the expression vector mentioned herein comprises a promoter capable of overexpressing the nucleotide sequence encoding an aldose-1-epimerase. Examples of such promoters include the GPD promoter, the TEF promoter and the ADP promoter. Preferred promoters which may be used to overexpress an aldose-1-epimerase can be any of the regulatory elements controlling the expression of nucleotide sequences encoding proteins involved in glycolysis and glucose fermentation.

As used herein, the term “higher rate” in the phrase “a transformed microorganism capable of converting an aldopentose to a ketopentose at a higher rate than the equivalent microorganism prior to transformation” refers to a transformed microorganism which is capable of decreasing the amount of aldopentose in a culture medium such that the reduction in the amount of aldopentose in the culture medium is at least 5%, 10%, 20% or 25% more per cell than that of the equivalent microorganism prior to transformation when cultured under the same culture conditions for a given time period within the exponential growth phase.

The term "exponential growth phase" is used in the normal sense of the art - e.g. the microorganisms are dividing at a constant rate such that the total number of microorganisms doubles with each division. The skilled person can readily determine the lag phase (the period
5 during which the cells adjust to a new environment before the onset of exponential growth), the exponential growth phase, the stationary phase (where the rate of cell division equals the rate of cell death, hence viable cell number remains constant) and the death phase (where the viable count declines) for any microorganism for a given set of culture conditions.

10 The term "equivalent microorganism prior to transformation" as used herein refers to the microorganism prior to transformation with a nucleotide sequence that encodes an aldose-1-epimerase or prior to transformation with a nucleotide sequence that causes the upregulation (e.g. overexpression) of an aldose-1-epimerase.

15 As used herein the term "the conversion of an aldopentose to a ketopentose" refers to, for example, the conversion of the aldopentose xylose to the ketopentose xylulose; the conversion of the aldopentose arabinose to the ketopentose xylulose; the conversion of the aldopentose arabinose to the ketopentose ribulose; the conversion of the aldopentose lyxose to the ketopentose xylulose; and the conversion of the aldopentose ribose to the ketopentose ribulose.

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In a preferred aspect, the ketopentose is xylulose.

The term "a transformed microorganism capable of converting an aldopentose to a ketopentose" as used herein refers to a microorganism which comprises one or more polynucleotide
25 sequences encoding one or more polypeptides involved in the conversion of an aldopentose to a ketopentose. Examples of polypeptides capable of converting an aldopentose to a ketopentose include xylose isomerase, arabinose isomerase, D-lyxose isomerase, and ribose isomerase; the combination of xylose reductase and xylulose reductase, the combination of arabinose reductase, L-arabitol 4-dehydrogenase, L-xylulose reductase and D-xylulose reductase, and the
30 combination of D-lyxose reductase and D-arabinitol dehydrogenase. The polypeptide(s) may be endogenous and/or exogenous to said microorganism. The polypeptide(s) may be encoded by one or more expression vectors.

Preferably the aldopentose is selected from the group consisting of xylose, arabinose, ribose and lyxose. Preferably the aldopentose is xylose or arabinose. More preferably, the aldopentose is xylose.

- 5 The spontaneous conversion between α -aldopentose and β -aldopentose in microorganisms is slow. For example, the spontaneous conversion of β -D-xylose to α -D-xylose has a first-order K-value of about 0.094/min at physiologic temperature (Bailey *et al.*, 1969).

As used herein, the term "overexpress" in the phrase "a nucleotide sequence that causes the
10 microorganism to overexpress an aldose-1-epimerase" and "a promoter capable of overexpressing the nucleotide sequence encoding an aldose-1-epimerases" refers to an increase in expression from zero to a level of expression or going from a lower level of expression to a higher level of expression (e.g. upregulation) when the transformed microorganism is compared to the equivalent microorganism prior to transformation. Microorganisms overexpressing an
15 aldose-1-epimerase have an increased ability to catalyse the conversion between α -aldopentose and β -aldopentose.

Preferably said transformed microorganism which overexpresses an aldose-1-epimerase is able to catalyse the conversion of an α -aldopentose to a β -aldopentose at a rate which is at least 10%,
20 15%, 20% or 25% higher than an untransformed microorganism, measured in an assay where 1g of disrupted cell mass has been added to 50 ml of a freshly prepared, buffered, neutral solution containing 100 mM of the β -aldopentose, using the interconversion assay as described later herein.

25 Examples of microorganisms overexpressing an aldose-1-epimerase include: (i) microorganisms transformed with an expression vector encoding an aldose-1-epimerase (prior to transformation said microorganism was not capable of expressing the aldose-1-epimerase); and (ii) microorganisms transformed to upregulate the expression of an endogenous aldose-1-epimerase (prior to transformation said microorganism was capable of expressing said aldose-1-epimerase
30 for a given set of culture conditions during exponential growth but after transformation said microorganism is capable of expressing said aldose-1-epimerase at a higher level, in the same culture conditions, during exponential growth).

As used herein the term "higher growth rate" in the phrase "a transformed microorganism
35 capable of a higher growth rate in the presence of aldopentose than the equivalent

microorganism prior to transformation” refers to a transformed microorganism which is capable of an increased growth rate such that the time taken for a doubling in the number of microorganisms per ml during the exponential growth phase is at least 10%, 15%, 20% or 25% lower than that of the equivalent microorganism prior to transformation when cultured under the same culture conditions. In a preferred aspect, the microorganisms are cultured at their optimal growth temperature; and preferably the culture medium comprises between 1% and 4% aldopentose in addition to optimal amounts of salts, vitamins and other nutrients necessary for the microorganism.

10 The term “higher metabolism” as used herein in the phrase “a transformed microorganism capable of a higher metabolism of aldopentose than the equivalent microorganism prior to transformation” refers to a transformed microorganism which is capable of a metabolising aldopentose such that the consumed amount of the aldopentose in the culture medium is at least 10%, 20%, 25%, 30% or 35% higher per cell than that of the equivalent microorganism prior to transformation when cultured under the same culture conditions for a given time period within the exponential growth phase. In a preferred aspect, the microorganisms are cultured at their optimal growth temperature; and preferably the culture medium comprises between 1% and 4% aldopentose in addition to optimal amounts of salts, vitamins and other nutrients necessary for the microorganism.

20 Preferably the transformed microorganism according to the present invention has a rate of conversion of the aldopentose to the ketopentose which is at a higher level than the equivalent microorganism prior to transformation. The term “higher level” as used in this phrase refers to a transformed microorganism which is capable of a converting aldopentose such that the reduction in the amount of aldopentose in the culture medium is at least 5%, 10%, 20% or 25% more per cell than that of the equivalent microorganism prior to transformation when cultured under the same culture conditions for a given time period within the exponential growth phase.

30 The term “a nucleotide sequence encoding an aldose-1-epimerase” as used herein encompasses nucleotide sequences comprising regulatory sequences enabling the expression of the nucleotide sequence encoding an aldose-1-epimerase such as promoters and enhancers which may be natively or non-natively associated with the nucleotide sequence encoding an aldose-1-epimerase.

As used herein, the term "higher rate" in the phrase "a transformed microorganism capable of producing a biofuel at a higher rate in a culture medium than the equivalent microorganism prior to transformation" refers to a transformed microorganism capable of producing at least 10%, 20%, 25%, 30% or 35% more biofuel per cell than that of the equivalent microorganism prior to transformation when cultured under the same culture conditions for a given time period within the exponential growth phase. Preferably, the microorganisms are cultured at their optimal production circumstances (e.g. optimal oxygen pressure and stirring speed), and preferably the culture medium comprises between 2% and 6% aldopentose in addition to optimal amounts of salts, vitamins and other nutrients necessary for the microorganism.

10

Preferably, the method for producing a biofuel further comprises the step of obtaining the biofuel from the culture medium.

Transformed microorganism

15

As mentioned herein, the term "transformed microorganism" refers to a microorganism that has been genetically altered by recombinant DNA technology. The term "transformed" as used herein is synonymous with terms such as "transfected", "recombinant", "genetically engineered" and "genetically modified".

20

The term "transformed microorganism" in relation to the present invention includes any microorganism that comprises an expression vector(s) comprising the nucleotide sequence(s) mentioned herein and/or a promoter(s) that is capable of allowing the expression (in particular overexpression i.e. upregulation) of the nucleotide sequence(s) mentioned herein. In one embodiment the nucleotide sequence(s) is incorporated in the genome of the microorganism. In another embodiment, the promoter is incorporated in the genome of the microorganism. These features enable the transformed microorganism (when compared to equivalent microorganism prior to transformation) to (i) metabolise aldopentose sugars at a higher rate; and/or (ii) have a higher growth rate; and/or (iii) produce ketopentose at a higher rate; and/or (iv) produce a pentose derived compound at a higher rate; and/or (v) produce a biofuel at a higher rate.

25

The term "transformed microorganism" does not cover native nucleotide coding sequences in their natural environment when they are under the control of their native promoter which is also in its natural environment.

30

35

Therefore, the transformed microorganism of the present invention includes a microorganism comprising any one of, or combinations of, the nucleotide sequences coding for the enzymes mentioned herein, constructs comprising said nucleotide sequences, vectors comprising said nucleotide sequences, plasmids comprising said nucleotide sequences and expression vectors
5 comprising said nucleotide sequences.

Thus, a further embodiment of the present invention provides microorganisms transformed or transfected with a nucleotide sequence(s) that expresses the enzyme(s) mentioned herein. The microorganism will be chosen to be compatible with the vector and may be, for example,
10 bacterial, fungal or yeast cells.

Examples of suitable bacterial host organisms are gram positive or gram negative bacterial species.

15 Depending on the nature of the nucleotide sequence(s) encoding the enzyme(s) mentioned herein, eukaryotic hosts such as yeasts or other fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate.

The use of suitable microorganisms - such as yeast and fungal host cells - may provide for post-translational modifications (e.g. myristoylation, glycosylation, truncation, lapidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products mentioned herein.
20

Suitable microorganisms include bacteria, fungi and yeasts. Preferably the microorganism is a
25 yeast or a bacterium.

Preferably said transformed microorganism is a transformed yeast. Preferably said transformed yeast is derived from the genus *Saccharomyces*. More preferably said transformed yeast is *Saccharomyces cerevisiae*.
30

In another embodiment, preferably said transformed microorganism is a transformed bacterium. Preferably said transformed bacterium is derived from the genus *Zymomonas* or the genus *Zymobacter*. More preferably said transformed bacterium is *Zymomonas mobilis* or *Zymobacter palmae*.
35

In one embodiment, the transformed microorganism described herein metabolises aldopentose sugars at a higher rate when cultured in a culture medium comprising aldopentose than the equivalent microorganism prior to transformation.

- 5 In another aspect, the growth rate of the transformed microorganism described herein is higher when cultured in a culture medium comprising aldopentose than the equivalent microorganism prior to transformation.

- 10 In a further aspect, the transformed microorganism mentioned herein is capable of producing the ketopentose at a higher rate when cultured in a culture medium comprising aldopentose than the equivalent microorganism prior to transformation.

- 15 In another aspect, the transformed microorganism mentioned herein is capable of producing a biofuel at a higher rate when cultured in a culture medium comprising aldopentose than the equivalent microorganism prior to transformation.

- Microorganism may be transformed using techniques which are routine in the art such as electroporation (Sambrook *et al* 1989). Further, the presence of a sequence in a transformed microorganism may be determined by growth selection on suitable media which select for the growth of the transformed microorganism. Alternatively or in addition, the presence of inserted, heterologous DNA sequences may be determined by direct colony PCR using primers specifically designed for the inserted sequence. Such techniques are well known and routine in the art (see, for example, Sambrook *et al* 1989 and Ausubel *et al* 1995).

- 25 A transformed microorganisms according to the present invention may be used in combination with one or more transformed microorganisms according to the present invention.

- For example, one or more transformed microorganisms according to the present invention capable of converting D-xylose to D-xylulose may be used in combination with one or more microorganisms selected from the group consisting of: a transformed microorganism according to the present invention capable of converting L-arabinase to D-xylulose; a transformed microorganism according to the present invention capable of converting L-arabinase to L-ribulose; a transformed microorganism according to the present invention capable of converting D-lyxose to D-xylulose; and a transformed microorganism according to the present invention capable of converting D-ribose to D-ribulose.
- 30
- 35

In another example, one or more transformed microorganism according to the present invention capable of converting L-arabinase to D-xylulose may be used in combination with one or more microorganisms selected from the group consisting of: a transformed microorganism according to the present invention capable of converting D-xylose to D-xylulose; a transformed microorganism according to the present invention capable of converting L-arabinase to L-ribulose; a transformed microorganism according to the present invention capable of converting D-lyxose to D-xylulose; and a transformed microorganism according to the present invention capable of converting D-ribose to D-ribulose.

10

In another example, one or more transformed microorganism according to the present invention capable of converting L-arabinase to L-ribulose may be used in combination with one or more microorganisms selected from the group consisting of: a transformed microorganism according to the present invention capable of converting D-xylose to D-xylulose; a transformed microorganism according to the present invention capable of converting L-arabinase to D-xylulose; a transformed microorganism according to the present invention capable of converting D-lyxose to D-xylulose; and a transformed microorganism according to the present invention capable of converting D-ribose to D-ribulose.

In a further example, one or more transformed microorganism according to the present invention capable of converting D-lyxose to D-xylulose may be used in combination with one or more microorganisms selected from the group consisting of: a transformed microorganism according to the present invention capable of converting L-arabinase to D-xylulose; a transformed microorganism according to the present invention capable of converting L-arabinase to L-ribulose; a transformed microorganism according to the present invention capable of converting D-xylose to D-xylulose; and a transformed microorganism according to the present invention capable of converting D-ribose to D-ribulose.

In another further example, one or more transformed microorganism according to the present invention capable of converting D-ribose to D-ribulose may be used in combination with one or more microorganisms selected from the group consisting of: a transformed microorganism according to the present invention capable of converting L-arabinase to D-xylulose; a transformed microorganism according to the present invention capable of converting L-arabinase to L-ribulose; a transformed microorganism according to the present invention

capable of converting D-lyxose to D-xylulose; and a transformed microorganism according to the present invention capable of converting D-xylose to D-xylulose.

The transformed microorganisms according to the present invention may be used in combination with one or more further microorganisms. For example, one or more transformed microorganisms according to the present invention may be cultured in combination with at least one microorganism capable of producing, under certain culture conditions, one or more components selected from the list consisting of: ethanol, lactic acid, succinic acid, acetic acid, acetaldehyde, itaconic acid, cresol, 3-hydroxypropionic acid, poly-3-hydroxyalkanoates, protocatechuic acid, pyrocatechol, guaiacol, veratrol, vanillin, vanillic acid, vanillyl alcohol, muconic acid, adipic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-methoxybenzoic acid, 4-aminobenzoate, 4-hydroxyaniline, 4-methoxyaniline, quinol, anisole, phenol, anthranilic acid, 3-hydroxyanthranilate, 2,3-dihydroxybenzoic acid, 2-aminophenol, 1,4-cyclohexanedione and aromatic amino acids.

In a further aspect, there is provided a combination of (i) one or more transformed microorganisms according to the present invention and (ii) at least one further microorganism capable of producing, under certain culture conditions, one or more components selected from the list consisting of: ethanol, lactic acid, succinic acid, acetic acid, acetaldehyde, itaconic acid, cresol, 3-hydroxypropionic acid, poly-3-hydroxyalkanoates, protocatechuic acid, pyrocatechol, guaiacol, veratrol, vanillin, vanillic acid, vanillyl alcohol, muconic acid, adipic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-methoxybenzoic acid, 4-aminobenzoate, 4-hydroxyaniline, 4-methoxyaniline, quinol, anisole, phenol, anthranilic acid, 3-hydroxyanthranilate, 2,3-dihydroxybenzoic acid, 2-aminophenol, 1,4-cyclohexanedione and aromatic amino acids.

Additionally, the present invention provides an inoculum comprising one of the above-mentioned combinations.

Further, there is provided a culture medium comprising one of the above-mentioned combinations.

In addition, the present invention provides a kit comprising (i) an inoculum comprising one or more microorganisms according to the present invention and (ii) an inoculum comprising one or more further microorganisms mentioned herein.

Expression vector

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

5

In one aspect, the expression vector is incorporated into the genome of a suitable microorganism. The term "incorporated" preferably covers stable incorporation into the genome.

The nucleotide sequences mentioned herein may be present in a vector in which the nucleotide sequence is operably linked to regulatory sequences capable of providing for the expression of the nucleotide sequence by a suitable host microorganism.

10

The vectors are transformed into a suitable host microorganisms as described herein.

The choice of vector e.g. a plasmid, cosmid, or phage vector will often depend on the microorganism into which it is to be introduced.

15

The vectors for use herein may contain one or more selectable marker nucleotide sequences - such as a nucleotide sequence which confers antibiotic resistance e.g. ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Alternatively, the selection may be accomplished by co-transformation (as described in WO91/17243).

20

Vectors may be used *in vitro*, for example to transfect, transform, transduce or infect a host microorganism.

25

The vector may further comprise a nucleotide sequence enabling the vector to replicate in the host microorganism in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

In a preferred aspect, the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein comprises a nucleotide sequence encoding an aldose-1-epimerase.

30

Preferably an expression vector as mentioned herein comprises the nucleotide sequence encoding an aldose-1-epimerase.

35

Preferably the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein comprises at least one expression vector encoding xylose reductase and/or D-xylulose reductase.

- 5 In another preferred aspect, the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein comprises an expression vector encoding xylose reductase and an expression vector encoding D-xylulose reductase.

10 In a further aspect, preferably the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein comprises at least one expression vector encoding xylose isomerase.

In another aspect, preferably the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein comprises at least one expression vector encoding arabinose
15 reductase and/or L-arabitol 4-dehydrogenase and/or L-xylulose reductase and/or D-xylulose reductase.

In a further preferred aspect, the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein comprises an expression vector encoding arabinose reductase,
20 an expression vector encoding L-arabitol 4-dehydrogenase, an expression vector encoding L-xylulose reductase, and an expression vector encoding D-xylulose reductase.

In a further preferred aspect, the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein comprises an expression vector encoding L-arabinose
25 isomerase.

Preferably, in another aspect, the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein comprises at least one expression vector encoding L-arabinose isomerase and/or ribulokinase and/or ribulose phosphate 4-epimerase.

30

In a further preferred aspect, the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein comprises an expression vector encoding L-arabinose isomerase, an expression vector encoding ribulokinase, and an expression vector encoding ribulose phosphate 4-epimerase.

35

In another preferred aspect the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein comprises at least one expression vector encoding D-lyxose isomerase.

- 5 In a further preferred aspect the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein comprises an expression vector encoding D-ribose isomerase.

Preferably, in another aspect, the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein may further comprise at least one expression vector encoding
10 one or more enzymes selected from the group consisting of xylulokinase, D-ribulokinase, ribose-5-phosphate isomerase, ribulose-5-phosphate epimerase, transaldolase, transketolase and any other enzyme of the pentose phosphate pathway. Preferably said microorganism capable of converting an aldopentose to a ketopentose as mentioned herein further comprises at least one expression vector encoding xylulokinase and/or D-ribulokinase. More preferably, said
15 microorganism capable of converting an aldopentose to a ketopentose as mentioned herein further comprises at least one expression vector encoding xylulokinase. In another embodiment, preferably the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein further comprises at least one expression vector encoding ribulose-5-phosphate epimerase and/or ribose-5-phosphate isomerase and/or transaldolase and/or
20 transketolase.

In one aspect, an expression vector as mentioned herein, may further encode one or more enzymes selected from the group consisting of an aldose-1-epimerase, xylose reductase, D-xylulose reductase, xylose isomerase, arabinose reductase, L-arabitol 4-dehydrogenase, L-
25 xylulose reductase, L-arabinose isomerase, ribulokinase, ribulose phosphate 4-epimerase, D-lyxose isomerase, D-ribose isomerase, xylulokinase, D-ribulokinase, ribulose-5-phosphate epimerase, ribose-5-phosphate isomerase, transaldolase, and transketolase.

In one aspect, an expression vector as mentioned herein, may further encode one or more
30 enzymes selected from the group consisting of xylulokinase, D-ribulokinase, ribose-5-phosphate isomerase, D-ribulose-5-phosphate epimerase, transaldolase, transketolase and any other enzyme of the pentose phosphate pathway. In a preferred aspect an expression vector as described herein further encodes xylulokinase and/or D-ribulokinase. More preferably, said expression vector as mentioned herein further encodes xylulokinase. In another embodiment,
35 preferably expression vector as mentioned herein further encodes ribulose-5-phosphate

epimerase and/or ribose-5-phosphate isomerase and/or transaldolase and/or transketolase. In another more preferred embodiment, preferably expression vector as mentioned herein further encodes ribose-5-phosphate isomerase and/or transaldolase and/or transketolase.

- 5 In a preferred aspect, the microorganism capable of converting an aldopentose to a ketopentose as mentioned herein further comprises at least one expression vector encoding an aldose-1-epimerase.

Preferably an expression vector as mentioned herein further comprises a nucleotide sequence
10 encoding an aldose-1-epimerase.

Regulatory sequences

In some applications, the nucleotide sequence(s) mentioned herein is operably linked to a
15 regulatory sequence which is capable of providing for the expression of the nucleotide sequence, such as by the chosen microorganism. By way of example, the present invention covers the use of a vector comprising the nucleotide sequence(s) mentioned herein operably linked to such a regulatory sequence, i.e. the vector is an expression vector.

20 The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

25 The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

30 Enhanced expression of the nucleotide sequence(s) encoding the enzyme(s) mentioned herein may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions.

Preferably, the nucleotide sequence(s) mentioned herein is operably linked to at least a promoter.
35

Other promoters may even be used to direct expression of the polypeptide(s) mentioned herein.

Examples of suitable promoters for directing the transcription of the nucleotide sequence in a bacterial, fungal or yeast cell are well known in the art.

5

The promoter can additionally include features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box.

10 **Constructs**

The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes a nucleotide sequence mentioned herein directly or indirectly attached to a promoter.

15

An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the Sh1-intron or the ADH intron, intermediate the promoter and the nucleotide sequence(s) mentioned herein. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. In some cases, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild type gene promoter and when they are both in their natural environment.

20

The construct may even contain or express a marker, which allows for the selection of the genetic construct.

25

For some applications, preferably the construct comprises at least the nucleotide sequence(s) mentioned herein operably linked to a promoter.

Promoters

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As mentioned herein, in one aspect the present invention relates to a microorganism that has been transformed with a nucleotide sequence, such as a promoter, that causes the microorganism to overexpress an aldose-1-epimerase.

For instance, the promoter is inserted into the genome of a microorganism which enables the microorganism to overexpress (e.g. upregulate) an endogenous nucleotide sequence encoding an aldose-1-epimerase.

- 5 In another aspect, the promoter is operably linked to a nucleotide sequence in, for example, an expression vector.

In another aspect, the promoter is not repressed by the presence of glucose.

- 10 Examples of suitable promoters that could be used in microorganisms according to the present invention, such as *Saccharomyces cerevisiae*, include: the promoter of the glyceraldehyde-3-phosphate dehydrogenase (GPD) gene; the promoter of the alcohol dehydrogenase (ADH) gene; the promoter of the Thyrotrophic embryonic factor (TEF) gene. Examples of suitable promoters that could be used in *Zymomonas mobilis* and in *Zymobacter palmarum* include the *Zymomonas*
 15 Glyceraldehyde-3-phosphate dehydrogenase promoter (Conway *et al*, 1987) and the *Zymomonas* enolase promoter (Burnett *et al*, 1992).

- Preferred promoters which may be used to overexpress an aldose-1-epimerase can be any of the regulatory elements controlling the expression of nucleotide sequences encoding proteins
 20 involved in glycolysis and glucose fermentation. Examples include, but not are limited to:

- the P-pgi promoter capable of expressing the PGI1 gene said promoter comprises the nucleotide sequence between this gene and the open reading frame YBR197C;
- the P-tpi promoter capable of expressing the TPI1 gene said promoter comprises the nucleotide sequence between this gene and the open reading frame YDR051C;
- 25 the P-hxk promoter capable of expressing the HXK2 gene said promoter comprises the nucleotide sequence between this gene and the gene FZF1;
- the P-pfk promoter capable of expressing the PFK1 gene said promoter comprises the nucleotide sequence between this gene and the gene YAP1802;
- the P-eno promoter capable of expressing the ENO2 gene said promoter comprises the nucleotide sequence between this gene and the gene SPC97;
- 30 the P-tdh promoter capable of expressing the TDH3 gene said promoter comprises the nucleotide sequence between this gene and the gene PDX1;
- the P-fba promoter capable of expressing the FBA1 gene said promoter comprises the nucleotide sequence between this gene and the gene MPE1;

- the P-gpm promoter capable of expressing the GPM1 gene said promoter comprises the nucleotide sequence between this gene and the open reading frame YKL151C;
- the P-pdc promoter capable of expressing the PDC1 gene said promoter comprises the nucleotide sequence between this gene and the gene STU2; and,
- 5 the P-pgm promoter capable of expressing the PGM2 gene said promoter comprises the nucleotide sequence between this gene and the gene YKU80.

Biofuel

- 10 As used herein, the term "biofuel" refers to a fuel (e.g. a liquid fuel) suitable for use in (for example) combustion engines. Said biofuel is derived from biological matter comprising pentose sugars and/or from which pentose sugars can be derived by hydrolysis by enzymatic means and/or by acidic treatment. Preferably said pentose sugar is an aldopentose.
- 15 Plant materials - including plant waste comprising lignocellulosic material (for instance: cereal straw, such as wheat straw; sugar beet pulp; sugar cane bagasse; sorghum stover; Soya bean stover; maize stover; corn stover; wood-chips; and paper-pulp) and whole plants (such as those which are grown for energy purposes e.g. switchgrass) - are suitable sources for pentose sugars, in particular aldopentose sugars, for the present invention. Other suitable sources of plant
- 20 material include non-waste products (in other words, food and feed sources) such as sugar cane extract, sugar beet extract, sorghum, Soya beans, wheat starch and corn starch.

Preferably the biofuel mentioned herein comprises at least one alcohol.

- 25 In a preferred aspect, the alcohol is selected from the group consisting of methanol, ethanol, propanol and butanol. More preferably the biofuel comprises ethanol.

- Preferably, said biofuel is obtained (or obtainable) -in other words, extracted (or extractable) - from the culture medium in which a transformed microorganism according to the present
- 30 invention has been cultured under suitable conditions. Said biofuel may be obtained (or obtainable) from the culture medium using techniques which are routine in the art such as the removal of microorganism by centrifugation, isolation of the supernatant followed by distillation, and a further dehydration step to yield 99.5 % pure ethanol.

- 35 The biofuel may comprise one or more further biofuel components such as butanol.

The one or more further biofuel components may be admixed with the biofuel before and/or after the biofuel is obtained or extracted (obtainable or extractable) from a culture.

- 5 Alternatively or in addition, one or more further biofuel components may be produced by culturing a microorganism in a culture medium before and/or after and/or at the same time as a transformed microorganism according to the present invention is/has been cultured in a culture medium in order to produce the biofuel.
- 10 The present invention further provides a transportation fuel which comprises a biofuel produced using the microorganisms according to the present invention.

Ethanol used as a transportation fuel may serve two different purposes:

- (i) it can act as an oxygenated additive that raises the octane value and reduces emission in
15 ReFormulated Gasoline (RFG) (tetraethyl lead or MTBE replacement);
- (ii) it can act as a partial or full substitute for Regular Gasoline (RG) to reduce dependency on gasoline supply.

Anhydrous ethanol has an octane value of 130, and can be added in concentrations of 5–10%
20 (depending on the season) to Regular Gasoline obtained directly from refineries. Traditionally, tetra-ethyl lead has been used for octane boosting however, due to health issues, the use of lead has been banned almost worldwide. The addition of oxygenates to Regular Gasoline lowers the carbon monoxide emissions as well as other particles contributing to air pollution. Methyl *tert*-butyl ether (MTBE) was initially used as a oxygenate additive, however the occurrence of
25 MTBE contamination in drinking water aquifers has prompted some states to ban the use of this oxygenate. Ethanol is increasingly used worldwide as a replacement for MTBE as an oxygenate additive for the manufacturing of RFG.

Apart from serving as an oxygenate additive in the production of ReFormulated Gasoline,
30 ethanol can be used as a general substitute for regular gasoline. Cars can use E10 blends (10% added ethanol) without any modification of the engine.

Further, vehicles have been manufactured which can run on 100% ethanol – in other words, there is no requirement for a fossil based fuel.

The transformed microorganisms according to the present invention or the microorganisms prepared by a method according to the present invention are capable of producing a biofuel at a higher rate than the equivalent microorganism prior to transformation. As used here, the term “higher rate” refers to a transformed microorganism which is capable of producing in the culture medium at least 5%, 10%, 20%, 25%, 30% or 35% more biofuel (such as bioethanol) per cell than that of the equivalent microorganism prior to transformation when cultured under the same culture conditions for a given time period within the exponential growth phase.

Pentose derived compound

10

As used herein, the term “pentose derived compound” refers to any compound derived from a pentose sugar. The pentose derived compound may be derived from an aldopentose. The pentose derived compound may be derived from a ketopentose.

15 Examples of pentose derived compounds include, but are not limited to: ethanol, aromatic amino acids, cresol, itaconic acid, lactic acid, succinic acid, acetic acid, acetaldehyde, poly-3-hydroxyalkanoates, and 3-hydroxypropionic acid. Figure 1 shows other pentose derived compounds such as ketopentoses derived from aldopentoses. A pentose derived product may be converted to another product. For example, the ketopentose D-xylulose, derived from the aldopentose D-xylose, may be converted via the pentose phosphate pathway into ethanol.

20 Preferably said pentose derived compound is ethanol, lactic acid, succinic acid, acetic acid, acetaldehyde, itaconic acid, cresol, 3-hydroxypropionic acid, poly-3-hydroxyalkanoates, protocatechuic acid, pyrocatechol, guaiacol, veratrol, vanillin, vanillic acid, vanillyl alcohol, muconic acid, adipic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-methoxybenzoic acid, 4-aminobenzoate, 4-hydroxyaniline, 4-methoxyaniline, quinol, anisole, phenol, anthranilic acid, 3-hydroxyanthranilate, 2,3-dihydroxybenzoic acid, 2-aminophenol, 1,4-cyclohexanedione and aromatic amino acids.

30 Preferably said pentose derived compound is ethanol, cresol, itaconic acid, lactic acid, succinic acid, and 3-hydroxypropionic acid.

More preferably said pentose derived compound is ethanol.

35 **Culture medium**

The culture medium comprises at least one pentose. In a preferred aspect the culture medium comprises at least one aldopentose.

- 5 Preferably the transformed microorganisms are grown in the optimal culture medium for said microorganism. Using routine techniques, the optimal culture medium can be determined; in addition, the optimal growth conditions can be determined.

In one aspect said culture medium comprises about 1%, about 2%, about 4%, about 8%, about
10 15% or about 25% aldopentose before inoculation with the microorganism (i.e. at time zero).

Preferably said culture medium comprises the optimal amounts of salts, vitamins and other nutrients necessary for the microorganism.

- 15 The microorganisms are preferably cultured at their optimal growth temperature. The skilled person would have readily been able to determine the optimal temperature at which to culture microorganisms mentioned herein.

In one embodiment the microorganisms are cultured at about 20°C, 25°C, 30°C, 35°C, or 37°C.
20

In one embodiment the microorganisms are cultured at about 35°C to 39°C, preferably about 36°C to 38°C, more preferably at about 35.5°C to 37.5°C.

Preferably the microorganisms are cultured for about 3 hours, about 6 hours, about 15 hours,
25 about 24 hours, about 48 hours or about 96 hours.

In one aspect, the microorganism, in particular the transformed microorganism, is alcohol tolerant and/or acid tolerant.

- 30 The term "alcohol tolerant" in relation to the present invention refers to microorganisms which are capable of growth in a culture medium which comprises at least 2%, 5%, 10% or 15% alcohol.

As mentioned herein, the term "acid tolerant" refers to microorganisms which are capable of
35 growth in a culture medium which has a pH equal to or less than 6.5, 6.0, 5.0, 4.0 or 3.0.

In a preferred aspect, the culture medium is inoculated with at least 5×10^7 to 5×10^{11} cells per kg of culture medium, preferably 5×10^8 to 5×10^{10} cells per kg of culture medium, preferably 1×10^9 to 1×10^{10} cells per kg of culture medium and more preferably about 5×10^9 cells per kg of culture medium.

The terms "inoculum" and "starter culture" are interchangeable.

Sources of pentose sugars

10

Pentose sugars (in particular aldopentose sugars) are derived or derivable from plants.

15

Pentose sugars (in particular aldopentose sugars) may be derived from: plant materials typically used as food or feed sources (such as: sugar cane, sugar beet, sorghum, wheat and corn - which are starch-rich and sugar-rich plant materials); whole plants (such as those which are grown for energy purposes e.g. switchgrass); and, in particular, waste agricultural (plant) materials (such as: cereal straw, for instance, wheat straw; sugar beet pulp; bagasse, for instance, sugar cane bagasse; stovers, for instance, sorghum, Soya bean, maize or corn stovers; and wood chips).

20

Sources of pentose sugars for the culture medium described herein include lignocellulosic materials normally regarded as agricultural waste material. Stems, stalks and leaves contain lignocellulosic material. Sugar cane bagasses, corn stovers and wood chips (hemicellulose only) are three easily accessible sources of lignocellulosic material as these are already collected or stocked in large amounts for various reasons.

25

Lignocellulosic material consists primarily of long sugar chains. On average, two thirds of these sugars are hexose sugars, which are mainly present in cellulose, and one third of the sugars are pentose sugars present mainly in arabinoxylan polymers.

30

A significant amount of hemicellulose derived pentose sugar is xylose.

Lignocellulosic materials can be hydrolysed in order to release the hexose and/or pentose sugars in the long-chain sugars of the cellulose, hemicellulose and lignin.

Hydrolysis of lignocellulosic materials can be carried out by acidic treatment at elevated temperature. However, this treatment may generate sugar derived by-products that are toxic to the majority of microorganisms and prevent the conversion of the sugars to ethanol. Such toxic by-products (if generated) can be removed but this is generally uneconomical.

5

Alternatively, lignocellulosic materials can be hydrolysed using cellulose and hemicellulose hydrolyzing enzymes. Advantageously, this process avoids the generation of toxic side-products.

- 10 In a preferred aspect, the culture medium comprises material derived from one or more lignocellulosic materials which have been treated (examples of such treatment techniques include: steam treatment, steam explosion, wet oxidation, acid hydrolysis, alkaline wet oxidation and ammonia fibre expansion) to release hexose and/or pentose sugars. Preferably the lignocellulosic material is treated by an enzymatic hydrolysis process. Said hydrolysed
- 15 lignocellulosic material may be further treated in order to extract the sugars before the use of said extract in a culture medium.

Hydrolysis of lignocellulosic material

- 20 *Initial mechanical treatment:*

The lignocellulosic material is chopped into smaller pieces as and when deemed necessary. For example, wheat straw is cut into pieces of approximately 5 cm in length.

- 25 *Subsequent hydrothermal pretreatment:*

- The hydrothermal pretreatment of the lignocellulosic material may be carried out as a steam pretreatment followed by a washing step, thereby producing a fibre fraction and a liquid fraction. The fibre fraction contains more than 90% of the cellulose, the lignin originally
- 30 present in the cellulosic material, and some of the hemicelluloses. The liquid fraction contains sugars from the hemicelluloses (C5 sugars), more than 90% of alkali chlorides comprised in the lignocellulosic biomass, and the majority of fermentation inhibitors arising from pretreatment of lignocellulosic feedstock.

Typically, wheat straw is heated by steam to a temperature between 180 and 200°C with a residence time of 5-15 min. The pretreated biomass is unloaded from the pressure reactor and washed and pressed. Released steam is collected and reused for evaporation of the liquid fraction to feed molasses.

5

Enzymatic hydrolysis:

Subsequent hydrolysis of sugar polymers may be carried out by the addition of cellulases and hemicellulases, either prior to fermentation or during fermentation or both *inter alia* a simultaneous saccharification and fermentation process.

10

Hexose

Hexose sugars have 6 carbon atoms. Aldohexoses have an aldehyde at position 1, and ketohexoses having a ketone at position 2. Glucose is an example of an aldohexose. Fructose is an example of a ketohexose.

15

Pentose

Pentose sugars have 5 carbon atoms. Pentoses either have an aldehyde functional group in position 1 (aldopentoses) or a ketone functional group in position 2 (ketopentoses).

20

Aldopentose

Preferably said aldopentose is selected from the group consisting of xylose, arabinose, ribose and lyxose. More preferably the aldopentose is xylose or arabinose.

25

In a preferred aspect said aldopentose is xylose. More preferably said aldopentose is D-xylose.

Ketopentose

30

Preferably said ketopentose is xylulose or ribulose.

In one preferred aspect said ketopentose is xylulose. More preferably said ketopentose is D-xylulose.

35

In one preferred aspect said ketopentose is ribulose. More preferably said ribulose is D-ribulose.

5 **Conversion of an aldopentose a ketopentose**

Examples of an aldopentose which is converted to a ketopentose include but are not limited to: the conversion of xylose to xylulose; the conversion of arabinose to xylulose; the conversion of arabinose to ribulose; the conversion of lyxose to xylulose; the conversion of ribose to ribulose; 10 the conversion of D-xylose to D-xylulose; the conversion of L-arabinose to L-xylulose or D-xylulose; the conversion of L-arabinose to L-ribulose; the conversion of D-lyxose to D-xylulose; and the conversion of D-ribose to D-ribulose.

Without wishing to be bound by theory, typically the enzymes involved in the conversion of D- 15 xylose to D-xylulose in fungi are xylose reductase and D-xylulose reductase.

In bacteria, without wishing to be bound by theory, the enzyme which is typically involved in the conversion of D-xylose to D-xylulose is xylose isomerase.

20 Without wishing to be bound by theory, the enzymes which are generally involved in the conversion of L-arabinose to L-xylulose in fungi are arabinose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase and D-xylulose reductase.

In bacteria, without wishing to be bound by theory, typically the enzyme involved in the 25 conversion of L-arabinose to L-ribulose is L-arabinose isomerase.

Without wishing to be bound by theory, the enzyme which may be involved in the conversion of D-lyxose to D-xylulose in bacteria is lyxose isomerase.

30 Without wishing to be bound by theory, the enzyme which may be involved in the conversion of D-ribose to D-ribulose is D-ribose isomerase.

Enzymes

The enzyme nomenclature numbers (EC numbers) mentioned herein refer to the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the nomenclature and classification of enzymes published in 1992.

- 5 The enzymes mentioned herein can be produced by nucleotide sequences derived from a wide variety of sources. In one aspect, the nucleotide sequences encoding the enzymes mentioned herein may be derived or derivable from *Lactococcus lactis subsp. lactis*, *Geobacillus stearothermophilus*, *Enterococcus faecalis*, *Piromyces* sp, *Thermoanaerobacter thermohydrosulfuricus*, *Pichia stipitis*, or *Saccharomyces cerevisiae*.

10

Aldose-1-epimerase (EC 5.1.3.3)

An aldose-1-epimerase mentioned herein is capable of acting on the aldopentose.

- 15 Aldose-1-epimerase has the EC nomenclature number 5.1.3.3. Aldose-1-epimerase may be referred to as a mutarotase or an aldose mutarotase.

The term aldose-1-epimerase refers to an enzyme which is capable of converting an α -aldopentose to a β -aldopentose and *vice versa*.

20

- In a preferred embodiment the aldose-1-epimerase is encoded by a nucleotide sequence selected from the group consisting of: AAD20257 version 1, ABX75760 version 1, AAK05605 version 1, AAD20245 version 1, AAD20251 version 1, ABJ73095 version 1, ABI49935 version 1 and AAO80762 version 1 (NCBI accession numbers). More preferably the aldose-1-epimerase is
25 selected from the group consisting of: AAD20257, ABX75760, AAK05605, AAD20245 and AAD20251.

- Examples of aldose-1-epimerases suitable for use as described herein include aldose-1-epimerase encoded by: the nucleotide sequence of the *Lactococcus lactis* aldose-1-epimerase gene (NCBI accession number AAD20245 version 1); the nucleotide sequence of the
30 *Saccharomyces cerevisiae* GAL10 gene (in particular, the part encoding an amino acid sequence having mutarotase activity); and the nucleotide sequence of the *Saccharomyces cerevisiae* strain D0002 GAL10 gene (in particular, the part encoding an amino acid sequence having mutarotase activity).

35

Aldose reductase (EC 1.1.1.21)

Aldose reductase has the EC nomenclature number 1.1.1.21. Aldose reductase may be referred to as: polyol dehydrogenase, aldehyde reductase, ALR2, NADPH-aldopentose reductase,
5 NADPH-aldose reductase, alditol:NADP oxidoreductase or alditol:NADP⁺ 1-oxidoreductase.

The term aldose reductase refers to an enzyme which is capable of converting an alditol to an aldose and *vice versa*.

10 An aldose reductase may reduce more than one type of aldose. For example, the same enzyme may be capable of reducing both D-xylose and L-arabinose such an enzyme may thus be called aldose reductase or, it may be called more specifically after one of the substrates, e.g. xylose reductase.

15 Xylose reductase (EC 1.1.1.21)

In one embodiment, the aldose reductase is a xylose reductase. Xylose reductase has the EC nomenclature number 1.1.1.21.

20 The term xylose reductase refers to an enzyme which is capable of converting D-xylose to xylitol and *vice versa*.

A xylose reductase mentioned herein is capable of acting on D-xylose.

25 Examples of xylose reductases suitable for use as described herein include xylose reductase encoded by: the nucleotide sequence of *Pichia stipitis* xylose reductase gene (PsXR); the nucleotide sequence of *Pichia stipitis* strain DSM3651 xylose reductase gene (PsXR) - NCBI accession number X59465 version 1; the nucleotide sequence of *Candida tenuis* (said nucleotide sequence encoding xylose reductase can be obtained as described by Kavanagh *et al*, 2003); and
30 the nucleotide sequence of *Neurospora crassa* (said nucleotide sequence encoding xylose reductase can be obtained as described by Woodyer *et al*, 2005).

Arabinose reductase (EC 1.1.1.21)

In another embodiment, the aldose reductase is an arabinose reductase. Arabinose reductase has the EC nomenclature number 1.1.1.21.

The term arabinose reductase refers to an enzyme which is capable of converting L-arabinose to L-arabitol and *vice versa*.

An arabinose reductase mentioned herein is capable of acting on L-arabinose.

D-xylose reductases currently known in the art may also act on L-arabinose as a substrate with similar activity. Hence, the term L-arabinose reductase may also refer to enzymes which are classified as being D-xylose reductases, and the xylose reductases mentioned herein as suitable for introducing xylose metabolism are similarly suitable for use in introducing arabinose metabolism to a microorganism.

In a further embodiment, the aldose reductase may be capable of converting L-lyxose to L-arabitol and *vice versa*. In another embodiment, the aldose reductase may be capable of converting D-lyxose to D-arabitol and *vice versa*.

In another embodiment, the aldose reductase may be capable of converting ribose to ribitol (in particular D-ribose to D-ribitol) and *vice versa*.

Xylulose reductase (EC 1.1.1.9 and EC 1.1.1.10)

The term xylulose reductase encompasses D-xylulose reductase and L-xylulose reductase.

D-xylulose reductase (EC 1.1.1.9)

D-xylulose reductase has the EC nomenclature number 1.1.1.9. D-xylulose reductase may be referred to as xylitol dehydrogenase, xylitol-2-dehydrogenase, 2,3-*cis*-polyol(DPN)dehydrogenase (C3-5), NAD-dependent xylitol dehydrogenase, erythritol dehydrogenase or pentitol-DPN dehydrogenase.

The term D-xylulose reductase refers to an enzyme which is capable of converting xylitol to D-xylulose and *vice versa*.

A D-xylulose reductase mentioned herein is capable of acting on xylitol.

Examples of D-xylulose reductases suitable for use as described herein include D-xylulose reductase encoded by: the nucleotide sequence of *Pichia stipitis* D-xylulose gene (PsXDH); and
5 the nucleotide sequence of *Pichia stipitis* strain DSM3651 D-xylulose reductase gene (PsXDH) - NCBI accession number X55392 version 1.

L-xylulose reductase (EC 1.1.1.10)

10 L-xylulose reductase has the EC nomenclature number 1.1.1.10. L-xylulose reductase may be referred to as L xylitol dehydrogenase.

The term L-xylulose reductase refers to an enzyme which is capable of converting L-xylulose to xylitol and *vice versa*.

15

An L-xylulose reductase mentioned herein is capable of acting on L-xylulose.

A nucleotide sequence encoding L-xylulose reductase may be obtained from *Aspergillus niger* as described by Witteveen *et al* (1994) or from the yeast *Ambrosiozyma monospora* (Verho *et al*, 2004).
20

Xylulokinase (EC 2.7.1.17)

Xylulokinase has the EC nomenclature number 2.7.1.17. Xylulokinase may be referred to as D-
25 xylulokinase.

The term xylulokinase refers to an enzyme which is capable of converting D-xylulose to D-xylulose 5-phosphate and *vice versa*.

30 A xylulokinase mentioned herein is capable of acting on D-xylulose.

Examples of xylulokinases suitable for use as described herein include xylulokinase encoded by: the nucleotide sequence of *Pichia stipitis* xylulokinase gene (PsXKS); the nucleotide sequence of *Pichia stipitis* strain DSM3651 xylulokinase gene (PsXKS) - NCBI accession number
35 AF127802 version 1; the nucleotide sequence of *S. cerevisiae* xylulokinase gene (ScXKS); and

the nucleotide sequence of *S. cerevisiae* strain D0002 xylulokinase gene (ScXKS) - NCBI accession number X61377 version 1.

Xylose isomerase (EC 5.3.1.5)

5

Xylose isomerase has the EC nomenclature number EC 5.3.1.5. Xylose isomerase may be referred to as D-xylose isomerase, D-xylose ketoisomerase, or D-xylose ketol-isomerase.

10 The term xylose isomerase refers to an enzyme which is capable of converting D-xylose to D-xylulose and *vice versa*.

A xylose isomerase mentioned herein is capable of acting on D-xylose.

15 Examples of xylose isomerases suitable for use as described herein include xylose isomerase encoded by: the nucleotide sequence of *Piromyces* xylose isomerase gene (PmXI); the nucleotide sequence of *Piromyces* sp E2 xylose isomerase gene (PmXI) - NCBI accession number AJ249909 version 1; and the nucleotide sequence of *Thermoanaerobacter thermohydrosulfuricus* xylose isomerase gene (ThXI) - NCBI accession number D00756 version 1; SEQ ID No 2 and SEQ ID No 3.

20

D-arabinitol 4-dehydrogenase (EC 1.1.1.11)

D-arabinitol 4-dehydrogenase has the EC nomenclature number 1.1.1.11. D-arabinitol 4-dehydrogenase may be referred to as D-arabitol dehydrogenase or arabitol dehydrogenase.

25

The term D-arabinitol 4-dehydrogenase refers to an enzyme which is capable of converting D-arabinitol to D-xylulose and *vice versa*.

A D-arabinitol 4-dehydrogenase mentioned herein is capable of acting on D-arabinitol.

30

A suitable D-arabinitol 4-dehydrogenase and the corresponding gene is described by Cheng *et al* 2005.

L-arabinitol 4-dehydrogenase (EC 1.1.1.12)

35

L-arabinitol 4-dehydrogenase has the EC nomenclature number 1.1.1.12. L-arabinitol 4-dehydrogenase may be referred to as L-arabitol 4-dehydrogenase or pentitol-DPN dehydrogenase.

- 5 The term L-arabinitol 4-dehydrogenase refers to an enzyme which is capable of converting L-arabinitol to L-xylulose and *vice versa*.

An L-arabinitol 4-dehydrogenase mentioned herein is capable of acting on L-arabinitol.

- 10 A suitable L-arabinitol 4-dehydrogenase and the corresponding gene is described in Richard *et al* (2001).

L-arabinose isomerase (EC 5.3.1.4)

- 15 L-arabinose isomerase has the EC nomenclature number 5.3.1.4. L-arabinose isomerase may be referred to as L-arabinose ketol-isomerase.

The term L-arabinose isomerase refers to an enzyme which is capable of converting L-arabinose to L-ribulose and *vice versa*.

20

An L-arabinose isomerase mentioned herein is capable of acting on L-arabinose.

An example of a nucleotide sequence encoding L-arabinose isomerase is the nucleotide sequence which may be obtained from *Lactobacillus plantarum* strain NCIMB8826 (ATCC
25 14917) (gene described in NCBI accession code NC_004567 version 1).

Ribulokinase (EC 2.7.1.16)

- 30 Ribulokinase has the EC nomenclature number 2.7.1.16. Ribulokinase may be referred to as L-ribulokinase.

The term ribulokinase refers to an enzyme which is capable of converting (i) L-ribulose to L-ribulose 5-phosphate and *vice versa*; and/or (ii) D-ribulose to D-ribulose 5-phosphate and *vice versa*.

35

A ribulokinase mentioned herein is capable of acting on L-ribulose and/or D-ribulose.

A suitable nucleotide sequence encoding ribulokinase may be obtained from *Lactobacillus plantarum* strain NCIMB8826 (ATCC 14917) (gene described in NCBI accession code
5 NC_004567 version 1).

L-ribulose phosphate 4-epimerase (EC 5.1.3.4)

L-ribulose phosphate 4-epimerase has the EC nomenclature number 5.1.3.4. L-ribulose
10 phosphate 4-epimerase may be referred to as ribulose phosphate 4-epimerase, phosphoribulose isomerase, L-ribulose 5-phosphate 4-epimerase, AraD or L-Ru5P.

The term L-ribulose phosphate 4-epimerase refers to an enzyme which is capable of converting L-ribulose 5-phosphate to D-xylulose 5-phosphate and *vice versa*.

15

An L-ribulose phosphate 4-epimerase mentioned herein is capable of acting on L-ribulose 5-phosphate.

A suitable nucleotide sequence encoding L-ribulose phosphate 4-epimerase may be obtained
20 from *Lactobacillus plantarum* strain NCIMB8826 (ATCC 14917) (gene described in NCBI accession code NC_004567 version 1).

D-ribitol 4-dehydrogenase

25 D-ribitol 4-dehydrogenase has the EC nomenclature number 1.1.1.56. This enzyme may also be referred to as ribitol 2-dehydrogenase, adonitol dehydrogenase or ribitol dehydrogenase.

The term D-ribitol 4-dehydrogenase refers to an enzyme which is capable of converting ribitol to D-ribulose and *vice versa*.

30

A D-ribitol 4-dehydrogenase mentioned herein is capable of acting on D-ribitol.

A suitable D-ribitol 4-dehydrogenase and the corresponding gene is described by Dothie *et al*, 1985.

35

D-lyxose isomerase (EC 5.3.1.15)

D-lyxose isomerase has the EC nomenclature number 5.3.1.15. This enzyme may also be referred to as D-lyxose ketol-isomerase.

5

The term D-lyxose isomerase refers to an enzyme which is capable of converting D-lyxose to D-xylulose and *vice versa*.

A D-lyxose isomerase mentioned herein is capable of acting on D-lyxose.

10

A nucleotide sequence encoding a D-lyxose/L-ribose isomerase may be cloned from the organism *Acinetobacter* sp. strain DL-28 (Shimonishi and Izumori, 1996) or from *Aerobacter aerogenes* (Anderson and Allison, 1965).

15 Ribose isomerase (EC 5.3.1.20)

Ribose isomerase has the EC nomenclature number 5.3.1.20. This enzyme may also be referred to as D-ribose isomerase or D-ribose ketol-isomerase.

20 The term ribose isomerase refers to an enzyme which is capable of converting D-ribose to D-ribulose and *vice versa*.

A ribose isomerase mentioned herein is capable of acting on D-ribose.

25 D-ribose isomerase has been found in the organism *Mycobacterium smegmatis* (Izumori *et al*, 1975), from where it may be cloned.

Ribulose-5-phosphate 3-epimerase (EC 5.1.3.1)

30 Ribulose-5-phosphate 3-epimerase has the EC nomenclature number 5.1.3.1. This enzyme may also be referred to as: pentose-5-phosphate 3-epimerase, phosphoketopentose 3-epimerase, phosphoketopentose epimerase, phosphoribulose epimerase, ribulose-phosphate 3-epimerase; D-ribulose 5-phosphate epimerase; D-ribulose phosphate-3-epimerase; D-ribulose-5-P 3-epimerase; D-xululose-5-phosphate 3-epimerase; erythrose-4-phosphate isomerase; ribulose 5-
35 phosphate 3-epimerase; and xylulose phosphate 3-epimerase.

The term ribulose-5-phosphate 3-epimerase refers to an enzyme which is capable of converting D-ribulose 5-phosphate to D-xylulose 5-phosphate and *vice versa*.

- 5 Examples of ribulose-5-phosphate 3-epimerase suitable for use as described herein include ribulose-5-phosphate 3-epimerase encoded by: the nucleotide sequence of the *S. cerevisiae* *RPE1* gene; the nucleotide sequence of the *S. cerevisiae* strain D0002 *RPE1* gene; the nucleotide sequence of NCBI accession code NP_012414 version 1; and the ribulose-5-phosphate isomerase from *P. stiptitis* that can be found in NCBI accession number NP_012414
10 version 1.

Ribose-5-phosphate isomerase (EC 5.3.1.6)

- Ribose-5-phosphate isomerase has the EC nomenclature number 5.3.1.6. This enzyme may also
15 be referred to as phosphopentoisomerase, phosphopentoseisomerase, phosphopentosisomerase, phosphoriboisomerase, ribose 5-phosphate epimerase, ribose phosphate isomerase, 5-phosphoribose isomerase, or D-ribose-5-phosphate ketol-isomerase.

- The term ribose-5-phosphate isomerase refers to an enzyme which is capable of converting D-
20 ribose 5-phosphate to D-ribulose 5-phosphate and *vice versa*.

- Examples of ribose-5-phosphate isomerase suitable for use as described herein include ribose-5-phosphate ketol-isomerase encoded by: the nucleotide sequence of the *S. cerevisiae* *RKII* gene; the nucleotide sequence of the *S. cerevisiae* strain D0002 *RKII* gene; the nucleotide sequence of
25 NCBI accession code X94335 version 1; the nucleotide sequence of NCBI accession code NP_014738 version 1, and the ribose-5-phosphate isomerase from *P. stiptitis* that can be found in accession NC_009043 version 1.

Transketolase (EC 2.2.1.1)

30

Transketolase has the EC nomenclature number 2.2.1.1. This enzyme may also be referred to as glycolaldehydetransferase.

The term transketolase refers to an enzyme which is capable of converting sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate to D-ribose 5-phosphate + D-xylulose 5-phosphate and *vice versa*.

- 5 Examples of transketolases suitable for use as described herein include transketolase encoded by: the nucleotide sequence of the *Saccharomyces cerevisiae* *TKL1* gene; the nucleotide sequence of the *Saccharomyces cerevisiae* strain D0002 *TKL1* gene; the nucleotide sequence of NCBI accession code X73224 version 1; the nucleotide sequence of NCBI accession code NP_015399 version 1, and the transketolase from *P. stiptitis* that can be found in accession
10 CP000496 version 1.

Transaldolase (EC 2.2.1.2)

- Transaldolase has the EC nomenclature number 2.2.1.2. This enzyme may also be referred to as
15 dihydroxyacetone transferase, dihydroxyacetone synthase, formaldehyde transketolase or glycerone transferase.

- The term transaldolase refers to an enzyme which is capable of converting sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate to D-erythrose 4-phosphate + D-fructose 6-phosphate
20 and *vice versa*.

- Examples of transaldolases suitable for use as described herein include transaldolase encoded by: the nucleotide sequence of the *Saccharomyces cerevisiae* *TAL1* gene; the nucleotide sequence of the *Saccharomyces cerevisiae* strain D0002 *TAL1* gene; the nucleotide sequence of
25 NCBI accession code X15953 version 1; the nucleotide sequence of NCBI accession code NP_013458 version 1, and the transaldolase from *P. stiptitis* that can be found in accession CP000502 version 1.

Aldopentose assay

- 30 The amount of an aldopentose in a solution (such as a culture medium) may be determined colorimetrically by the phloroglucinol method, as described by Ebert *et al* (A Simplified, Colorimetric Micromethod for Xylose in Serum or Urine, with Phloroglucinol, 1979, *Clin. Chem.* **25**, no.8, pp. 1440-1443).

The colour reagent consists of 0.5 g phloroglucinol (1,3,5 trihydroxybenzene), 100 ml glacial acetic acid and 10 ml conc. HCl. 50 μ l of sample is added 950 μ l of the colour reagent. The mixture is heated to 100°C for 4 minutes, and the absorbance of the mixture is read at 554 nm. The amount of an aldopentose in the sample is determined according to a standard curve made with the same aldopentose as standard. This method may be used to determine the amount of xylose, arabinose, lyxose and ribose in a culture medium.

Interconversion assay

The ability of microorganisms to catalyse the conversion between α -aldopentose and β -aldopentose can be assayed by various methods. For example, the interconversion between the alpha and the beta anomer of aldoses may be followed by NMR, for example as explained by Ryu *et al* (2004), or by a coupled assay, where the activity of an enzyme, specific for one of the anomers, is followed, as described by Brahma and Bhattacharyya (2004).

Ketopentose assay

The amount of a ketopentose in a solution (such as a culture medium) may be determined colorimetrically by the cysteine-carbazole method as described by Zacharias Dische and Ellen Borenfreund (1951; J. Biol. Chem. 192 (2): 583).

Ethanol assay

The amount of the biofuel as ethanol, in a solution (such as a culture medium) may be determined by the use of a commercially available ethanol assay, the K-ETOH Kit, manufactured and sold by Megazyme International, Bray Business Park, Bray, Co. Wicklow, Ireland; or it may be determined by, for example, the use of gas chromatography.

Pentose phosphate pathway

Ketopentoses are converted into ethanol via the pentose phosphate pathway. An example of this is shown in Figure 4.

Examples of enzymes involved in the pentose phosphate pathway include: transketolase, transaldolase, ribose-5-phosphate ketol-isomerase and ribulose-5-phosphate 3-epimerase.

In one embodiment the microorganism according to the present invention has also been transformed to express or overexpress one or more enzymes involved in the pentose phosphate pathway.

5

In one embodiment the microorganism according to the present invention has also been transformed with one or more nucleotide sequences that cause the microorganism to overexpress one or more enzymes involved in the pentose phosphate pathway. For example, a promoter is inserted into the genome of a microorganism which enables the microorganism to overexpress an endogenous nucleotide sequence encoding an enzyme involved in the pentose phosphate pathway.

10

In another embodiment the microorganism has been transformed with one or more nucleotide sequences encoding one or more enzymes involved in the pentose phosphate pathway. For example, the microorganism is transformed with an expression vector comprising a nucleotide sequence encoding one or more enzymes involved in the pentose phosphate pathway.

15

Preferably the expression vector mentioned herein comprises one or more promoters capable of overexpressing one or more nucleotide sequences encoding one or more enzymes involved in the pentose phosphate pathway. Examples of such promoters include the GPD promoter, the TEF promoter and the ADP promoter. Preferred promoters which may be used to overexpress one or more nucleotide sequences encoding one or more enzymes involved in the pentose phosphate pathway can be any of the regulatory elements controlling the expression of nucleotide sequences encoding proteins involved in glycolysis and glucose fermentation.

20

25

As used herein, the term "overexpress" in the phrase "one or more nucleotide sequences that cause the microorganism to overexpress one or more enzymes involved in the pentose phosphate pathway" and "one or more promoters capable of overexpressing one or more nucleotide sequences encoding one or more enzymes involved in the pentose phosphate pathway" refers to an increase in expression from zero to a level of expression or going from a lower level of expression to a higher level of expression (e.g. upregulation) when the transformed microorganism is compared to the equivalent microorganism prior to transformation. Microorganisms overexpressing one or more enzymes involved in the pentose phosphate pathway have an increased ability to catalyse the conversion of a ketopentose (such as xylulose 5-phosphate) to a biofuel (such as ethanol).

30

35

Preferably said transformed microorganism which overexpresses one or more enzymes involved in the pentose phosphate pathway is able to catalyse the conversion of a ketopentose (such as xylulose 5-phosphate) to a biofuel (such as ethanol) by a rate which is at least 10%, 15%, 20% or 25% higher than an untransformed microorganism.

Examples of microorganisms overexpressing one or more enzymes involved in the pentose phosphate pathway include: (i) microorganisms transformed with one or more expression vectors encoding one or more of transketolase, transaldolase, ribose-5-phosphate ketol-isomerase and ribulose-5-phosphate 3-epimerase; and (ii) microorganisms transformed to upregulate the expression of one or more endogenous nucleotide sequences encoding one or more of transketolase, transaldolase, ribose-5-phosphate ketol-isomerase and ribulose-5-phosphate 3-epimerase (prior to transformation said microorganism was capable of expressing one or more of these enzymes for a given set of culture conditions during exponential growth but after transformation said microorganism is capable of expressing one or more of these enzymes at a higher level, in the same culture conditions, during exponential growth).

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention.

EXAMPLES

General recombinant DNA methodology techniques

The present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example Sambrook et al, 1989; Ausubel, F. M. *et al.* 1995; Roe *et al* 1996; Gait, 1984; and Lilley and Dahlberg, 1992. Each of these general texts is herein incorporated by reference.

The present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; M. J. Gait (Editor), 1984,

Oligonucleotide Synthesis: A Practical Approach, Irl Press; and, D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA Methods in Enzymology*, Academic Press.

5 Example 1a - Construction of synthetic mutarotase gene (aldose-1-epimerase) of *Lactococcus lactis* based on NCBI accession code AAD20245 version 1.

The entire *L. lactis* aldose-1-epimerase gene (LIMR) was synthesized and assembled by Geneart AG (Regensburg, Germany). Codon usage in the sequence was optimised based on the yeast codon usage table from the Kazusa codon usage database (Nakamura *et al.*, 2000).
 10 Flanking the open reading frame, a restriction-site for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the stop-codon, were included in the synthetic construct. The integrity of the LIMR synthetic gene was determined by sequencing of both strands. The nucleotide sequence of LIMR including the flanking restriction-sites is identified as SEQ.ID.NO. 1 (i.e. AAD20245) and the amino acid sequence encoded by this nucleotide
 15 sequence is identified as SEQ ID No 47. The harbouring plasmid was named 0717050pGA14.

Example 1b - Construction of synthetic xylose isomerase gene of *Piromyces sp. E2* based on NCBI accession code AJ249909 version 1.

The entire *Piromyces* xylose isomerase gene (PmXI) was synthesized and assembled by Geneart AG (Regensburg, Germany). Codon usage in the sequence was optimised based on the
 20 yeast codon usage table from the Kazusa codon usage database (Nakamura *et al.*, 2000). Flanking the open reading frame, a restriction-site for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the stop-codon, were included in the synthetic construct. The integrity of the PmXI synthetic gene was determined by sequencing of both strands. The nucleotide sequence of PmXI including flanking restriction-sites is identified as SEQ.ID.NO. 2
 25 and the amino acid sequence encoded by this nucleotide sequence is identified as SEQ ID No 48. The harbouring plasmid was named 0717049pGA15.

Example 1c - Construction of synthetic xylose isomerase (XylA) gene of *Thermoanaerobacter thermohydrosulfuricus* based on NCBI accession code D00756 version 1.

30 The entire *T. thermohydrosulfuricus* xylose isomerase gene (ThXI) was synthesized and assembled by Geneart AG (Regensburg, Germany). Codon usage in the sequence was optimised based on the yeast codon usage table from the Kazusa codon usage database (Nakamura *et al.*, 2000). Flanking the open reading frame, a restriction-site for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the stop-codon, were included in the synthetic
 35 construct. The integrity of the ThXI synthetic gene was determined by sequencing of both

strands. The nucleotide sequence of ThXI including flanking restriction-sites is identified as SEQ.ID.NO. 3 and the amino acid sequence encoded by this nucleotide sequence is identified as SEQ ID No 49. The harbouring plasmid was named 0717046pGA14.

Example 2a - TOPO cloning of the D-xylulokinase gene from the *Pichia stipitis* strain DSM3651 based on NCBI accession code AF127802 version 1.

The entire *P. stipitis* D-xylulokinase gene (PsXKS) was PCR amplified from DNA obtained from the strain DSM3651 using the primers identified by SEQ.ID.NO. 4 and SEQ.ID.NO. 5. A restriction-site for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the stop-codon was introduced flanking the PsXKS gene. As template, DNA from the *P. stipitis* strain was used in a concentration of 0.2ng/μl PCR-reaction. PCR was performed at 30 cycles of 30 seconds at 96°C, 30 seconds at 50°C, and 150 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 1% low melt agarose gel and a 1891bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named pCR-Blunt 2 P.stip XKS.

Example 2b - TOPO cloning of the D-xylose reductase gene from the *Pichia stipitis* strain DSM3651 based on NCBI accession code X59465 version 1.

The entire *P. stipitis* xylose reductase gene (PsXR) was PCR amplified from DNA obtained from the strain DSM3651, using the primers identified by SEQ.ID.NO. 6 and SEQ.ID.NO. 7. A restriction-site for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the stop-codon was introduced flanking the PsXR gene. As template, DNA from the *P. stipitis* strain was used in a concentration of 0.2ng/μl PCR-reaction. PCR was performed at 30 cycles of 30 seconds at 96°C, 30 seconds at 50°C, and 150 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 1% low melt agarose gel and a 976bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named pCR-Blunt 2 P.stip XR.

Example 2c - TOPO cloning of the xylitol dehydrogenase (D-xylulose reductase gene from the *Pichia stipitis* strain DSM3651 based on NCBI accession code X55392 version 1.

The entire *P. stipitis* xylitol dehydrogenase gene (PsXDH) was PCR amplified from DNA obtained from the strain DSM3651, using the primers identified by SEQ.ID.NO. 8 and

SEQ.ID.NO. 9. A restriction-site for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the stop-codon was introduced flanking the PsXDH gene. As template, DNA from the *P. stipitis* strain was used in a concentration of 0.2ng/μl PCR-reaction. PCR was performed at 30 cycles of 30 seconds at 96°C, 30 seconds at 50°C, and 150 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 1% low melt agarose gel and a 1108bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named pCR-Blunt 2 P.stip XDH.

Example 2d - TOPO cloning of the L-arabinose isomerase gene (EC 5.3.1.4) from the *Lactobacillus plantarum* strain NCIMB8826 (ATCC 14917) based on NCBI accession code NC_004567 version 1.

The entire *L. plantarum* L-arabinose isomerase gene (LpAraA) is PCR amplified from DNA obtained from the strain ATCC14917, using the primers identified by SEQ.ID.NO. 10 and SEQ.ID.NO. 11. A restriction-site for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the stop-codon are introduced flanking the LpAraA gene. As template, DNA from the *L. plantarum* strain is used in a concentration of 0.2ng/μl PCR-reaction. PCR is performed at 30 cycles of 30 seconds at 96°C, 30 seconds at 55°C, and 60 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product is electrophoretically separated on a 0.7% low melt agarose gel and a 1444 bp fragment is isolated. The DNA fragment is TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 2e - TOPO cloning of the L-ribulokinase gene (EC 2.7.1.16) from the *Lactobacillus plantarum* strain NCIMB8826 (ATCC 14917) based on NCBI accession code NC_004567 version 1.

The entire *L. plantarum* L-ribulokinase gene (LpAraB) is PCR amplified from DNA obtained from the strain ATCC14917, using the primers identified by SEQ.ID.NO. 12 and SEQ.ID.NO. 13. A restriction-site for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the stop-codon are introduced flanking the LpAraB gene. As template, DNA from the *L. plantarum* strain is used in a concentration of 0.2ng/μl PCR-reaction. PCR is performed at 30 cycles of 30 seconds at 96°C, 30 seconds at 55°C, and 60 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product is electrophoretically separated on a

0.7% low melt agarose gel and a 1618 bp fragment is isolated. The DNA fragment is TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 2f - TOPO cloning of the L-ribulose-5-phosphate 4-epimerase gene (EC 5.1.3.4) from the *Lactobacillus plantarum* strain NCIMB8826 (ATCC 14917) based on NCBI accession code NC_004567 version 1.

The entire *L. plantarum* L-ribulose-5-phosphate 4-epimerase gene (LpAraD) is PCR amplified from DNA obtained from the strain ATCC14917, using the primers identified by SEQ.ID.NO. 14 and SEQ.ID.NO. 15. A restriction-site for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the stop-codon are introduced flanking the LpAraD gene. As template, DNA from the *L. plantarum* strain was used in a concentration of 0.2ng/μl PCR-reaction. PCR was performed at 30 cycles of 30 seconds at 96°C, 30 seconds at 55°C, and 60 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product is electrophoretically separated on a 0.7% low melt agarose gel and a 745 bp fragment is isolated. The DNA fragment is TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 2g - TOPO cloning of the GAL10 gene from the *Saccharomyces cerevisiae* strain D0002 based on NCBI accession number code Z35888 version 1.

The entire *S. cerevisiae* GAL10 gene (ScGAL10) was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 16. and SEQ.ID.NO. 17. A restriction-site for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the stop-codon were introduced flanking the ScGAL10 gene. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2ng/μl PCR-reaction. PCR was carried out for 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 120 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 0,7% low melt agarose gel and a 2116bp fragment isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named ScGAL-a23a.

Example 2h - TOPO cloning of a truncated GAL10 gene [nucleotides 1084 – 2100] from the *Saccharomyces cerevisiae* strain D0002 based on NCBI accession code Z35888 version 1.

The N-terminal truncated *S. cerevisiae* GAL10 gene (ScGAL10Δ) was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 18, and SEQ.ID.NO. 17. A restriction-site for NheI, proximal to a ATG-start codon encoded by the primer and a restriction-site for XhoI, distal to the stop-codon were introduced flanking the ScGAL10Δ gene. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2ng/μl PCR-reaction. PCR was carried out for 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 120 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 0,7% low melt agarose gel and a 1036bp fragment isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid used for the transformation of *E. coli* TOP10. The plasmid was named ScGALΔ-a24a.

Example 3a - Construction of plasmid PmXI-8a containing the *Piromyces* xylose isomerase (PmXI) gene under control of the GPD promoter and the CYC1 terminator from *S. cerevisiae*.

The *E. coli*/*S. cerevisiae* high-copy shuttle vector P426-GPD (Mumberg *et al.*, 1995) was digested with SpeI and XhoI and the resulting termini were dephosphorylated with alkaline phosphatase. Similarly, the DNA fragment encoding the *Piromyces* xylose isomerase gene was released from the vector 0717049pGA15 (described in Example 1b) by digestion with NheI and XhoI. The resulting linearized plasmid P426-GPD and the DNA fragment encoding PmXI were electrophoretically separated on a 1% low melt agarose gel and isolated. The two DNA fragments were ligated together resulting in the plasmid named PmXI-8a.

Example 3b - Construction of plasmid ThXI-5a containing the *T. thermohydrosulfuricus* xylose isomerase (ThXI) gene under control of the GPD promoter and the CYC1 terminator from *S. cerevisiae*.

The *E. coli*/*S. cerevisiae* high-copy shuttle vector P426-GPD (Mumberg *et al.*, 1995) was digested with SpeI and XhoI and the resulting termini were dephosphorylated with alkaline phosphatase. Similarly, the DNA fragment encoding the *T. thermohydrosulfuricus* xylose isomerase gene was released from the vector 0717046pGA14 (described in Example 1c) by digestion with NheI and XhoI. The resulting linearized plasmid P426-GPD and the DNA fragment encoding ThXI were electrophoretically separated on a 1% low melt agarose gel and isolated. The two DNA fragments were ligated together resulting in the plasmid named ThXI-5a.

Example 3c - Construction of plasmid PsXKS-14a containing the *P. stipitis* D-xylulokinase (PsXKS) gene under control of the GPD promoter and the CYC1 terminator from *S. cerevisiae*.

The *E. coli/S. cerevisiae* high-copy shuttle vector P425-GPD (Mumberg *et al.*, 1995) was digested with SpeI and XhoI and the resulting termini were dephosphorylated with alkaline phosphatase. Similarly, the DNA fragment encoding the *P. stipitis* xylulose kinase gene was released from the vector pCR-Blunt 2 P.stip XKS (described in Example 2a), by digestion with NheI and XhoI. The resulting linearized plasmid P425-GPD and the DNA fragment encoding PsXKS were electrophoretically separated on a 1% low melt agarose gel and isolated. The two DNA fragments were ligated together resulting in the plasmid named PsXKS-14a.

Example 3d - Construction of plasmids PsXR-24a and PsXR-25 containing the *P. stipitis* xylose reductase (PsXR) gene under control of the GPD promoter and the CYC1 terminator from *S. cerevisiae*.

The *E. coli/S. cerevisiae* high-copy shuttle vector P424-GPD and the similar low-copy shuttle vector P414-GPD (Mumberg *et al.*, 1995) were digested with SpeI and XhoI and the resulting termini were dephosphorylated with alkaline phosphatase. Similarly, the DNA fragment encoding the *P. stipitis* xylose reductase gene was released from the vector pCR-Blunt 2 P.stip XR (described in Example 2b), by digestion with NheI and XhoI. The resulting linearized plasmids P424-GPD and P414-GPD and the DNA fragment encoding PsXR were electrophoretically separated on a 1% low melt agarose gel and isolated. The linearized plasmid P424-GPD was ligated together with the PsXR fragment resulting in the plasmid named PsXR-24a. Likewise, the linearized plasmid P414-GPD was ligated together with the PsXR fragment resulting in the plasmid named PsXR-25a.

Example 3e - Construction of plasmid PsXDH-11a containing the *P. stipitis* xylitol dehydrogenase (PsXDH) gene (xylulose reductase) under control of the GPD promoter and the CYC1 terminator from *S. cerevisiae*.

The *E. coli/S. cerevisiae* high-copy shuttle vector P426-GPD (Mumberg *et al.*, 1995) was digested with SpeI and XhoI and the resulting termini were dephosphorylated with alkaline phosphatase. Similarly, the DNA fragment encoding the *P. stipitis* xylulose dehydrogenase gene was released from the vector pCR-Blunt 2 P.stip XDH (described in Example 2c), by digestion with NheI and XhoI. The resulting linearized plasmid P426-GPD and the DNA fragment encoding PsXDH were electrophoretically separated on a 1% low melt agarose gel and isolated. The two DNA fragments were ligated together resulting in the plasmid named PsXDH-11a.

Example 3f - Construction of plasmids L1MR-36a, L1MR-38a and L1MR-40a containing the *L. lactis* mutarotase gene (L1MR) gene (aldose-1-epimerase) under control of various promoters and the CYC1 terminator from *S. cerevisiae*.

The *E. coli/S. cerevisiae* low-copy shuttle vectors P413-GPD, P413-ADH and P413-TEF (Mumberg *et al.*, 1995) were digested with SpeI and XhoI and the resulting termini were dephosphorylated with alkaline phosphatase. Similarly, the DNA fragment encoding the *L. lactis* aldose-1-epimerase gene (L1MR) gene was released from the vector 0717050pGA14 (described in Example 1a) by digestion with NheI and XhoI. The resulting linearized plasmids P413-GPD, P413-ADH and P413-TEF and the DNA fragment encoding L1MR were electrophoretically separated on a 1% low melt agarose gel and isolated. The linearized plasmid P413-GPD was ligated together with the L1MR fragment resulting in the plasmid named L1MR-36a. Likewise, the linearized plasmid P413-ADH was ligated together with the L1MR fragment resulting in the plasmid named L1MR-38a. Finally, the linearized plasmid P413-TEF was ligated together with the L1MR fragment resulting in the plasmid named L1MR-40a.

P413-GPD comprises the promoter from the gene TDH3 encoding Glyceraldehyde-3-phosphate dehydrogenase, (also known as GAPDH) isozyme 3; P413-ADH comprises the promoter from the gene ADH1 encoding Alcohol dehydrogenase I; and P413-TEF comprises the promoter from the gene TEF2 encoding Translational elongation factor EF-1 alpha.

Example 3g - Construction of a yeast expression plasmid containing the *L. plantarum* L-arabinose isomerase gene (LpAraA) gene under control of the GPD promoter and the CYC1 terminator from *S. cerevisiae*.

The *E. coli/S. cerevisiae* high-copy shuttle vector P426-GPD (Mumberg *et al.*, 1995) is digested with SpeI and XhoI and the resulting termini is dephosphorylated with alkaline phosphatase. Similarly, the DNA fragment encoding the *L. plantarum* L-arabinose isomerase gene (LpAraA) is released from the vector described in Example 2d, by digestion with NheI and XhoI. The resulting linearized plasmid P426-GPD and the DNA fragment encoding LpAraA are electrophoretically separated on a 0.7% low melt agarose gel and isolated. The two DNA fragments are ligated together and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 3h - Construction of a yeast expression plasmid containing the *L. plantarum* L-ribulokinase gene (LpAraB) gene under control of the ADH promoter and the CYC1 terminator from *S. cerevisiae*.

The *E. coli/S. cerevisiae* high-copy shuttle vector P425-ADH (Mumberg *et al.*, 1995) is digested with SpeI and XhoI and the resulting termini is dephosphorylated with alkaline phosphatase. Similarly, the DNA fragment encoding the *L. plantarum* L-ribulokinase gene

(LpAraB) is released from the vector described in Example 2e, by digestion with NheI and XhoI. The resulting linearized plasmid P425-ADH and the DNA fragment encoding LpAraB are electrophoretically separated on a 0.7% low melt agarose gel and isolated. The two DNA fragments are ligated together and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 3i - Construction of a yeast expression plasmid containing the *L. plantarum* L-ribulose-5-phosphate 4-epimerase gene (LpAraD) gene under control of the GPD promoter and the CYC1 terminator from *S. cerevisiae*.

The *E. coli/S. cerevisiae* high-copy shuttle vector P424-GPD (Mumberg *et al.*, 1995) is digested with SpeI and XhoI and the resulting termini is dephosphorylated with alkaline phosphatase. Similarly, the DNA fragment encoding the *L. plantarum* L-ribulose-5-phosphate 4-epimerase gene (LpAraD) is released from the vector described in Example 2f, by digestion with NheI and XhoI. The resulting linearized plasmid P424-GPD and the DNA fragment encoding LpAraD are electrophoretically separated on a 0.7% low melt agarose gel and isolated. The two DNA fragments are ligated together and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 3j - Construction of a yeast expression plasmid containing the *S. cerevisiae* bifunctional aldose-1-epimerase/UDP galactose 4-epimerase gene (ScGAL10) gene under control of the ADH promoter and the CYC1 terminator from *S. cerevisiae* for overexpression of aldose-1-epimerase.

The *E. coli/S. cerevisiae* low-copy shuttle vector P413-ADH (Mumberg *et al.* (1995) Gene 156, p.119-122) is digested with SpeI and XhoI and the resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the DNA fragment encoding the *S. cerevisiae* bifunctional aldose-1-epimerase/UDP galactose 4-epimerase gene (ScGAL10) is released from the vector ScGAL-a23a (described in Example 2g) by digestion with NheI and XhoI. The resulting linearized plasmid P413-ADH and the DNA fragment encoding ScGAL10 are electrophoretically separated on a 0.7% low melt agarose gel and thereafter isolated. The linearized plasmid P413-ADH is ligated together with the ScGAL10 fragment resulting in the yeast expression plasmid.

Example 3k - Construction of a yeast expression plasmid containing the mutarotase part of the *S. cerevisiae* aldose-1-epimerase/UDP galactose 4-epimerase gene (ScGAL10 Δ) gene under control of the ADH promoter and the CYC1 terminator from *S. cerevisiae* for overexpression of aldose-1-epimerase.

The gene GAL10 is known to be a bifunctional enzyme containing an epimerase part catalysing the conversion of UDP-galactose to UDP-glucose as well as a mutarotase part catalysing the conversion of α -galactose to β -galactose and *vice versa* Majumdar *et al.*, 2004.

The *E. coli*/*S. cerevisiae* low-copy shuttle vector P413-ADH (Mumberg *et al.* (1995) Gene 156, p.119-122) is digested with SpeI and XhoI and the resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the DNA fragment encoding the *S. cerevisiae* mutarotase part of the bifunctional aldose-1-epimerase/UDP galactose 4-epimerase gene (ScGAL10 Δ) is released from the vector ScGAL Δ -a24a (described in Example 2h) by digestion with NheI and XhoI. The resulting linearized plasmid P413-ADH and the DNA fragment encoding ScGAL10 Δ are electrophoretically separated on a 0.7% low melt agarose gel and thereafter isolated. The linearized plasmid P413-ADH is ligated together with the ScGAL10 Δ fragment resulting in the yeast expression plasmid.

Example 4a - Construction of *S. cerevisiae* strains containing the PmXI-8a and the PsXKS-14a plasmids together with, either the LIMR-36a, LIMR-38a, LIMR-40a or the empty P413-CYC plasmids (Mumberg *et al.*, 1995).

200ng each of the plasmids were combined and used for the transformation of *S. cerevisiae* yeast strain BY4741 (Euroscarf, Germany) by means of electroporation using the Biorad Gene Pulser II system (Biorad, USA) according to the manufacturer's instructions. Yeast cells were made competent according to a standard protocol (Becker, D. M. and Guarente, 1991). Selection for clones transformed with all three plasmids was done on solid synthetic complete dropout media omitting uracil, histidine and leucine and supplemented with 2% D-glucose (SC-Ura, His, Leu) (Rose *et al.*, 1990). Medium-size primary clones were restreaked on SC-Ura, His, Leu and one colony each of the following was isolated: strain T0062 transformed with the plasmids LIMR-36a, PmXI-8a and PsXKS-14a; strain T0063 with the plasmids LIMR-38a, PmXI-8a and PsXKS-14a; strain T0065 with the plasmids LIMR-40a, PmXI-8a and PsXKS-14a; and finally strain T0067 with the plasmids P413-CYC, PmXI-8a and PsXKS-14a (these plasmid are described in Example 3; P413-CYC is the empty plasmid described in Mumberg *et al* 1995).

Example 4b - Measurements of D-xylose metabolism by growth curves of the yeast strains T0062, T0063, T0065 and T0067.

Changes in the rate of D-xylose metabolism were measured as alterations in the growth rate of the xylose metabolising yeast strains. The four strains were initially adapted to D-xylose metabolism. Each strain was inoculated individually in liquid Synthetic Complete dropout media omitting uracil, histidine and leucine supplemented with 2% D-xylose and 0.2% D-glucose (SCX(+0.2% D-glc) – Ura, His, Leu). The cultures were incubated for one week at

30°C in a shaker running at 225 RPM. After one week, each culture was used to re-inoculate new cultures with liquid Synthetic Complete dropout media omitting uracil, histidine and leucine supplemented with 2% D-xylose and 0.02% D-glucose (SCX(+0.02% D-glc) – Ura, His, Leu). These cultures were incubated for a further week as described above. Growth experiments were initiated by inoculation of SCX-Ura, His, Leu supplemented with 2% D-xylose at an initial cell titre of OD600 = 0.006/ml. These cultures were incubated as described above. Aliquots were sampled four times with intervals of 24 hours, the optical density, OD600 was measured and a growth curve was determined for each of the four strains. The doubling time (i.e. the time taken for a doubling in the number of microorganisms per ml during the exponential growth phase) was determined, using the time interval of 24 – 96 hours following the initial lag phase. The following growth data could be determined for the four strains:

Yeast strain	Specific growth rate (μ) h ⁻¹	Final OD600 ml ⁻¹
T0067	0.058	0.110
T0065	0.070	0.215
T0063	0.079	0.441
T0062	0.088	0.524

These results show an increase in the growth rate of about 20% or more in the strains when the aldose-1-epimerase is co-expressed together with the xylose isomerase and D-xylokinase compared to the isogenic strain not express mutarotase. Compare strains T0056, T0063 and T0062 with T0067 that does not expressing the mutarotase.

In particular, strain T0062 showed an increase in the growth rate of more than 50%. In terms of assimilated carbon, more than 4 fold increase in biomass was achieved in that recombinant yeast strain. Furthermore, the use of different promoters controlling the aldose-1-epimerase, in otherwise isogenic strains, demonstrates an increase carbon flux as a result of an increased promoter strength. This shows that a bottleneck exist prior to the isomerisation of D-xylose in the metabolic pathway of D-xylose catabolism.

Example 5a - Construction of *S. cerevisiae* strains containing the ThXI-5a and the PsXKS-14a plasmids together with, either the LIMR-36a, or the empty P413-CYC plasmids (Mumberg *et al.*, 1995).

200ng each of the plasmids were combined and used for the transformation of *S. cerevisiae* yeast strain BY4741 (Euroscarf, Germany) by means of electroporation using the Biorad Gene Pulser II system (Biorad, USA) according to the manufacturer's instruction. Yeast cells were made competent according to a standard protocol (Becker, D. M. and Guarente, 1991). Selection for clones transformed with all three plasmids was done on solid synthetic complete dropout media omitting uracil, histidine and leucine and supplemented with 2% D-

glucose (SC-Ura, His, Leu) (Rose *et al.*, 1990). Medium-size primary clones were restreaked on SC-Ura, His, Leu and one colony each of the following was isolated: strain T0085 transformed with the plasmids LMR-36a, ThXI-5a and PsXKS-14a; and strain T0086 with the plasmids P413-CYC, ThXI-5a and PsXKS-14a.

5 Example 5b - Measurements of D-xylose metabolism by growth curves of the yeast strains T0085, and T0086.

Changes in the rate of D-xylose metabolism were measured as alterations in the growth rate of the xylose metabolising yeast strains. The two strains were initially adapted to D-xylose metabolism. Each strain was inoculated individually in liquid Synthetic Complete dropout media omitting uracil, histidine and leucine supplemented with 2% D-xylose and 0.2% D-glucose (SCX(+0.2% D-glc) – Ura, His, Leu). The cultures were incubated for one week at 30°C in a shaker running at 225 RPM. After one week, each culture was used to re-inoculate new cultures with liquid Synthetic Complete dropout media omitting uracil, histidine and leucine supplemented with 2% D-xylose and 0.02% D-glucose (SCX(+0.02% D-glc) – Ura, His, Leu). These cultures were incubated for a further week as described above. Growth experiments were initiated by inoculation of SCX-Ura, His, Leu supplemented with 2% D-xylose at an initial cell titre of OD600 = 0.006/ml. These cultures were incubated as described above. Aliquots were sampled four times with intervals of 24 hours, the optical density, OD600 was measured and a growth curve was determined for each of the four strains. The doubling time was determined, using the time interval of 24 – 96 hours following the initial lag phase. The following growth data could be determined for the two strains:

Yeast strain	Specific growth rate (μ) h ⁻¹	Final OD600 ml ⁻¹
T0086	0.056	0.101
T0085	0.067	0.172

This demonstrates that an increase in the growth-rate of about 20% can be achieved when the aldose-1-epimerase is co-expressed together with xylose isomerase and D-xylulokinase compared to the isogenic strain not express mutarotase. Compare strain T0085 with strain T0086 which does not express the mutarotase.

Example 6a - Construction of *S. cerevisiae* strains containing the PsXR-24a (or the PsXR-25), the PsXDH-11a, and the PsXKS-14a plasmids together with, either the LMR-38a, or the empty P423-CYC plasmids (Mumberg *et al.*, 1995).

200ng each of the plasmids were combined and used for the transformation of *S. cerevisiae* yeast strain Y07202 (Euroscarf, Germany) by means of electroporation using the Biorad Gene Pulser II system (Biorad, USA) according to the manufacturer's instruction. Yeast cells were made competent according to a standard protocol (Becker and Guarente, 1991).

Selection for clones transformed with all four plasmids simultaneous was done on solid synthetic complete dropout media omitting uracil, histidine, leucine and tryptophan and supplemented with 2% D-glucose (SC-Ura, His, Leu, Trp) (Rose *et al.*, 1990). Medium-size primary clones were restreaked on SC-Ura, His, Leu, Trp and one colony each of the following
 5 were isolated: strain T0114 transformed with the plasmids LIMR-38a, PsXR-24a, PsXDH-11a and PsXKS-14a; strain T0117 with the plasmids P423-CYC, PsXR-24a, PsXDH-11a and PsXKS-14a; strain T0123 with the plasmids LIMR-38a, PsXR-25a, PsXDH-11a and PsXKS-14a; and strain T0126 with the plasmids P423-CYC, PsXR-25a, PsXDH-11a and PsXKS-14a.

**Example 6b - Measurements of D-xylose metabolism by growth curves of the yeast strains
 10 T0114, T0117, T0123 and T0126.**

Changes in the rate of D-xylose metabolism were measured as alterations in the growth rate of the xylose metabolising yeast strains. The four strains were initially adapted to D-xylose metabolism. Each strain was inoculated individually in liquid Synthetic Complete dropout media omitting uracil, histidine, leucine and tryptophan supplemented with 2% D-xylose and
 15 0.2% D-glucose (SCX(+0.2% D-glc) – Ura, His, Leu, Trp). The cultures were incubated for one week at 30°C in a shaker running at 225 RPM. After one week, each culture was used to re-inoculate new cultures with liquid Synthetic Complete dropout media omitting uracil, histidine, leucine and tryptophan supplemented with 2% D-xylose and 0.02% D-glucose (SCX(+0.02% D-glc) – Ura, His, Leu, Trp). These cultures were incubated for a further week as described above.
 20 Growth experiments was initiated by inoculation of SCX-Ura, His, Leu, Trp supplemented with 2% D-xylose at an initial cell titre of OD600 = 0.006/ml. These cultures were incubated as described above. Aliquots were sampled five times with intervals of 24 hours, the optical density, OD600 was measured and a growth curve was determined for each of the four strains. The doubling time was determined, using the time interval of 24 – 120 hours following the
 25 initial lag phase. The following growth data could be determined for the four strains:

Yeast strain	Specific growth rate (μ) h ⁻¹	Final OD600 ml ⁻¹
T0114	0.040	0.089
T0117	0.033	0.055
T0123	0.039	0.084
T0126	0.034	0.059

This demonstrates that an increase in growth-rate of more than 10% can be achieved when the aldose-1-epimerase is co-expressed together with xylose reductase, xylulose dehydrogenase and D-xylulokinase compared to the isogenic strain not expressing mutarotase. Compare strain T0114 with strain T0117 which does not express the mutarotase and strain
 30 T0123 with strain T126 which does not express the mutarotase.

No significant differences between the strains expressing the *P. stipitis* xylose reductase on either a high copy (strain T0114) or a low copy plasmid (strain T0123) could be measured.

Example 7a - Construction of *S. cerevisiae* strains containing the LpAraA, LpAraB and LpAraD plasmids together with, either the LIMR-36a or the empty P413-CYC plasmid
 5 (Mumberg *et al.*, 1995).

200ng each of the plasmids LpAraA, LpAraB and LpAraD (described in Example 3g, 3h and 3i) are combined with either LIMR-36a (described in Example 3f) or with the empty plasmid P413-CYC (Mumberg *et al.*, 1995) and used for the transformation of *S. cerevisiae* yeast strain Y07202 (Euroscarf, Germany) by means of electroporation using the Biorad Gene
 10 Pulser II system (Biorad, USA) according to the manufacturer's instruction. Yeast cells are made competent according to a standard protocol (Becker, D. M. and Guarente, 1991). Selection for clones transformed with all four plasmids is carried out on solid synthetic complete dropout media omitting uracil, histidine, leucine and tryptophan and supplemented with 2% D-glucose (SC-Ura, His, Leu, Trp) (Rose *et al.*, 1990). Medium-size primary clones are restreaked on SC-
 15 Ura, His, Leu, Trp and one colony each of the yeast strains carrying either the aldose-1-epimerase (LIMR) gene or the empty vector P413-CYC is isolated.

Example 7b - Measurements of L-arabinose metabolism by growth curves of the yeast strains described in Example 7a.

Changes in the rate of L-arabinose metabolism are measured as alterations in the growth
 20 rate of the arabinose metabolising yeast strains. The two strains (described in Example 7a) are initially adapted to L-arabinose metabolism. Each strain is inoculated individually in liquid Synthetic Complete dropout media omitting uracil, histidine, leucine and tryptophan supplemented with 2% L-arabinose and 0.2% D-glucose (SCA(+0.2% D-glc) – Ura, His, Leu, Trp). The cultures are incubated for one week at 30°C in a shaker running at 225 RPM. After
 25 one week, each culture is used to re-inoculate new cultures with liquid Synthetic Complete dropout media omitting uracil, histidine, leucine and tryptophan supplemented with 2% L-arabinose and 0.02% D-glucose (SCA(+0.02% D-glc) – Ura, His, Leu, Trp). These cultures are incubated for a further week as described above. Growth experiments are initiated by inoculation of SCA-Ura, His, Leu, Trp supplemented with 2% L-arabinose at an initial cell titre
 30 of OD600 = 0.006/ml. These cultures are incubated as described above. Aliquots are sampled five times with intervals of 24 hours, the optical density, OD600 are measured and growth curves are determined for the two strains. The doubling times are determined, using the time interval of 24 – 120 hours following the initial lag phase.

An increase in growth-rate and in accumulated yeast biomass is achieved when the
 35 aldose-1-epimerase is co-expressed together with L-arabinose isomerase, (LpAraA) L-

ribulokinase (LpAraB) and L-ribulose-5-phosphate 4-epimerase (LpAraD) compared to the isogenic strain not expressing a mutarotase.

Example 8a - Construction of a *S. cerevisiae* strain containing the PmXI-8a and the PsXKS-14a plasmids together with the plasmid encoding ScGAL10 under control of the ADH promoter for overexpression of aldose-1-epimerase.

200ng each of the three plasmids is combined and used for the transformation of *S. cerevisiae* yeast strain BY4741 (Euroscarf, Germany) by means of electroporation using the Biorad Gene Pulser II system (Biorad, USA) according to the manufacturer's instruction. Yeast cells are made competent according to a standard protocol (Becker and Guarente 1991). Selection for clones transformed with all three plasmids is accomplished on solid synthetic complete dropout media omitting uracil, histidine and leucine and supplemented with 2% D-glucose (SC-Ura, His, Leu) (Rose. et. al. 1990). Clones comprising all three plasmids will grow on SC-Ura, His, Leu.

Example 8b - Construction of a *S. cerevisiae* strain containing the PmXI-8a and the PsXKS-14a plasmids together with the plasmid encoding ScGAL10 Δ under control of the ADH promoter for overexpression of aldose-1-epimerase.

200ng each of the three plasmids is combined and used for the transformation of *S. cerevisiae* yeast strain BY4741 (Euroscarf, Germany) by means of electroporation using the Biorad Gene Pulser II system (Biorad, USA) according to the manufacturer's instruction. Yeast cells are made competent according to a standard protocol (Becker and Guarente 1991). Selection for clones transformed with all three plasmids is accomplished on solid synthetic complete dropout media omitting uracil, histidine and leucine and supplemented with 2% D-glucose (SC-Ura, His, Leu) (Rose et. al. (1990). Clones comprising all three plasmids will grow on SC-Ura, His, Leu.

Example 8c - Measurements of D-xylose metabolism by growth curves of the yeast strains described in Example 8a and 8b.

Changes in the rate of D-xylose metabolism are measured as alterations in the growth rate of the xylose metabolising yeast strains. The two strains are initially adapted to D-xylose metabolism. First, by individual inoculation in liquid Synthetic Complete dropout media omitting uracil, histidine and leucine supplemented with 2% D-xylose and 0.2% D-glucose (SCX(+0.2% D-glc) – Ura, His, Leu) and incubation for one week at 30°C in a shaker running at 225 RPM. Thereafter, each culture is used for re-inoculation of new cultures in liquid Synthetic Complete dropout media omitting uracil, histidine and leucine supplemented with 2% D-xylose and 0.02% D-glucose (SCX(+0.02% D-glc) – Ura, His, Leu). Again, each culture is incubated for one week at 30°C in a shaker running at 225 RPM. Growth experiments are

initiated by inoculation of SCX-Ura, His, Leu supplemented with 2% D-xylose at an initial cell titre of OD600 = 0.006/ml. Strain T0067 (transformed with the plasmids P413-CYC, PmXI-8a and PsXKS-14a), described in Example 4a, may be included in the experiment, serving as an isogenic control for the measurement of growth without the ScGAL10 or the ScGAL10Δ gene present. Each of the three cultures is incubated as described above and aliquots are sampled four times with intervals of 24 hours. The optical density, OD600 is measured and, based on that, the growth curve for each strain is determined. The doubling time is determined, using the time interval of 24 – 96 hours following the initial lag phase. The increase in specific growth rate and in accumulated yeast biomass are demonstrated in these two yeast strains, expressing either ScGAL10 or ScGAL10Δ, compared to the isogenic T0067 strain not transformed with a heterologous yeast GAL10 expression derivative (e.g. ScGAL10 or ScGAL10Δ).

Example 9a - TOPO cloning of a Left Flanking Region for the stable integration of expression construct into the yeast Rdn1 locus (LFR_{Rdn1}) based on NCBI accession code DQ130086 version 1.

The *S. cerevisiae* DNA fragment allowing for the stable integration into the RDN37-2 part of RDN1 (LFR_{Rdn1}) was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 19 and SEQ.ID.NO. 20. A restriction-site for PmeI, proximal to the DNA fragment and a restriction-site for Sall, distal to DNA fragment was introduced flanking the LFR_{Rdn1} piece. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/μl PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 20 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 1.0 % low melt agarose gel and a 433 bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named Rdn1-L-a15c(+).

Example 9b - TOPO cloning of a Right Flanking Region for the stable integration of expression construct into the yeast Rdn1 locus (RFR_{Rdn1}) based on NCBI accession code DQ130089 version 1.

The *S. cerevisiae* DNA fragment allowing for the stable integration into the RDN37-2 part of RDN1 (RFR_{Rdn1}) was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 21 and SEQ.ID.NO. 22. A restriction-site for XhoI, proximal to the DNA fragment and a restriction-site for PmeI, distal to DNA fragment was introduced flanking the RFR_{Rdn1} piece. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/μl PCR-reaction. PCR was performed at 35 cycles of 30

seconds at 96°C, 30 seconds at 57°C, and 20 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 1.0 % low melt agarose gel and a 503 bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named Rdn1-R-a16b(+).

Example 9c - TOPO cloning of a Left Flanking Region for the stable integration of expression construct into the yeast MIG1 locus (LFR_{Mig1-2}) based on NCBI accession code Z72557 version 1.

The *S. cerevisiae* DNA fragment allowing for the stable integration into the MIG1 locus (LFR_{Mig1-2}) was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 23 and SEQ.ID.NO. 24. A restriction-site for PmeI, proximal to the DNA fragment and a restriction-site for Sall, distal to DNA fragment was introduced flanking the LFR_{Mig1-2} piece. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/μl PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 20 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 1.0 % low melt agarose gel and a 507 bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named Mig1-L2-a19h(+).

Example 9d - TOPO cloning of a Right Flanking Region for the stable integration of expression construct into the yeast MIG1 locus (RFR_{Mig1-2}) based on NCBI accession code Z72556 version 1.

The *S. cerevisiae* DNA fragment allowing for the stable integration into the MIG1 locus (RFR_{Mig1-2}) was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 25 and SEQ.ID.NO. 26. A restriction-site for XhoI, proximal to the DNA fragment and a restriction-site for PmeI, distal to DNA fragment was introduced flanking the RFR_{Mig1-2} piece. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/μl PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 20 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 1.0 % low melt agarose gel and a 500 bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO

vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named Mig1-R2-a20a(-).

Example 10a - Construction of a yeast integrative plasmid containing the *S. cerevisiae* Left and right flanking regions of the Rdn1 locus (LFR_{Rdn1}+ RFR_{Rdn1}) allowing for homologous recombination into the yeast genome.

The plasmid Rdn1-L-a15c(+) (described in Example 9a) was digested with XhoI and PsmOMI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid Rdn1-R-a16b(+) (described in Example 9b) was digested with XhoI and NotI. The resulting linearized plasmid Rdn1-L-a15c(+) and the DNA fragment containing RFR_{Rdn1} originating from the plasmid Rdn1-R-a16b(+) was electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid Rdn1-L-a15c(+) was ligated together with the RFR_{Rdn1} DNA fragment and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named Rdn1-LR-b5a.

Example 10b - Construction of a yeast integrative plasmid containing the *S. cerevisiae* Left and right flanking regions of the Mig1 locus (LFR_{Mig1-2}+ RFR_{Mig1-2}) allowing for homologous recombination into the yeast genome.

The plasmid Mig1-L2-a19h(+) (described in Example 9c) was digested with NotI and XbaI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid Mig1-R2-a20a(-) (described in Example 9d) was digested with SpeI and NotI. The resulting linearized plasmid Mig1-L2-a19h(+) and the DNA fragment containing RFR_{Mig1-2} originating from the plasmid Mig1-R2-a20a(-) was electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid Mig1-L2-a19h(+) was ligated together with the RFR_{Mig1-2} DNA fragment and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named Mig1-LR2-b7a.

Example 11 - Construction of *loxP* flanked antibiotic marker gene cassettes with either the *kanMX* or the *nat1* gene.

The plasmid pUG6 (Güldener *et al.*, 1996) was digested with Xba and XhoI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmids pAG25 (Goldstein and McCusker, 1999) and pUG6 was digested with SpeI and SalI. The resulting linearized plasmid pUG6 and the DNA fragments containing the *nat1* gene (originating from the plasmid pAG25) and the *kanMX* gene (originating from the plasmid pUG6) were electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid pUG6 was ligated together with the *nat1* encoding DNA fragment and the resulting plasmid was used for the transformation of *E. coli* TOP10. This plasmid was named pUG6R25-b2a. Similarly, the linearized plasmid pUG6 was ligated together

with the *kanMX* encoding DNA fragment and the resulting plasmid was used for the transformation of *E. coli* TOP10. This plasmid was named pUG6R6-b1a.

Example 12a - Construction of a yeast integrative plasmid containing the *S. cerevisiae* Left Flanking Region of the Rdn1 locus (LFR_{Rdn1}), the *loxP* flanked antibiotic marker gene *nat1* and the Right Flanking Region of the Rdn1 locus (RFR_{Rdn1}) allowing for homologous recombination into the yeast genome and subsequent selection using the antibiotic nourseothricin.

The plasmid Rdn1-LR-b5a (described in Example 10a) is digested with NotI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid pUG6R25-b2a (described in Example 11) is digested with NotI. The resulting linearized plasmid Rdn1-LR-b5a and the DNA fragment containing the *loxP* flanked *nat1* gene (originating from the plasmid pUG6R25-b2a) are electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid Rdn1-LR-b5a is ligated together with the *loxP* flanked *nat1* gene DNA fragment and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 12b - Construction of a yeast integrative plasmid containing the *S. cerevisiae* Left Flanking Region of the Mig1 locus (LFR_{Mig1-2}), the *loxP* flanked antibiotic marker gene *kanMX* and the Right Flanking Region of the Mig1 locus (RFR_{Mig1-2}) allowing for homologous recombination into the yeast genome and subsequent selection using the antibiotic G418.

The plasmid Mig1-LR2-b7a (described in Example 10b) is digested with NotI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid pUG6R6-b1a (described in Example 11) is digested with NotI. The resulting linearized plasmid Mig1-LR2-b7a and the DNA fragment containing the *loxP* flanked *kanMX* gene (originating from the plasmid pUG6R6-b1a) are electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid Mig1-LR2-b7a is ligated together with the *loxP* flanked *kanMX* gene DNA fragment and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 13a - TOPO cloning of a DNA fragment containing the transcription terminator of the open reading frame YBR197C and the promoter of the gene *PGII* based on NCBI accession code Z21487 version 1.

The *S. cerevisiae* DNA fragment (P-pgi) covering the region between the stop codon of the open reading frame YBR197C and ATG codon of the gene *PGII* was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 27 and SEQ.ID.NO. 28. A restriction-site for Sall, proximal to the DNA fragment and a

restriction-site for *AvrII*, distal to DNA fragment was introduced flanking the intergenic region. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/ μ l PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 30 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 0.7 % low melt agarose gel and a 1318 bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named P-pgi-ala(+).

Example 13b - TOPO cloning of a DNA fragment containing the transcription terminator of the open reading frame YDR051C and the promoter of the gene *TPII* based on NCBI accession code Z49209 version 1.

The *S. cerevisiae* DNA fragment (P-tpi) covering the region between the stop codon of the open reading frame YDR051C and ATG codon of the gene *TPII* was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 29 and SEQ.ID.NO. 30. A restriction-site for *Sall*, proximal to the DNA fragment and a restriction-site for *AvrII*, distal to DNA fragment was introduced flanking the intergenic region. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/ μ l PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 30 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 0.7 % low melt agarose gel and a 599 bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named P-tpi-a2d(+).

Example 13c - TOPO cloning of a DNA fragment containing the transcription terminator of the gene *YKU80* and the promoter of the gene *PGM2* based on NCBI accession code Z49702 version 1.

The *S. cerevisiae* DNA fragment (P-pgm) covering the region between the stop codon of gene *YKU80* and ATG codon of the gene *PGM2* was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 31 and SEQ.ID.NO. 32. A restriction-site for *Sall*, proximal to the DNA fragment and a restriction-site for *AvrII*, distal to DNA fragment was introduced flanking the intergenic region. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/ μ l PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 30 seconds at 72°C,

followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 0.7 % low melt agarose gel and a 710 bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named P-pgm-a10a(+).

Example 13d - TOPO cloning of a DNA fragment containing the transcription terminator of the gene *STU2* and the promoter of the gene *PDC1* based on NCBI accession code Z73217 version 1.

The *S. cerevisiae* DNA fragment (P-pdc) covering the region between the stop codon of gene *STU2* and ATG codon of the gene *PDC1* was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 33 and SEQ.ID.NO. 34. A restriction-site for *Sall*, proximal to the DNA fragment and a restriction-site for *AvrII*, distal to DNA fragment was introduced flanking the intergenic region. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/μl PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 30 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 0.7 % low melt agarose gel and a 971 bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named P-pdc-a9f(+).

Example 13e - TOPO cloning of a DNA fragment containing the transcription terminator of the gene *MPE1* and the promoter of the gene *FBA1* based on NCBI accession code Z28060 version 1.

The *S. cerevisiae* DNA fragment (P-fba) covering the region between the stop codon of gene *MPE1* and ATG codon of the gene *FBA1* was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 35 and SEQ.ID.NO. 36. A restriction-site for *Sall*, proximal to the DNA fragment and a restriction-site for *AvrII*, distal to DNA fragment was introduced flanking the intergenic region. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/μl PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 30 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 0.7 % low melt agarose gel and a 646 bp fragment was isolated. The DNA fragment was TOPO cloned into

the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named P-fba-a7b(+).

Example 13f - TOPO cloning of a DNA fragment containing the transcription terminator of the open reading frame YKL151C and the promoter of the gene *GPM1* based on NCBI accession code Z26877 version 1.

The *S. cerevisiae* DNA fragment (P-gpm) covering the region between the stop codon of the open reading frame YKL151C and ATG codon of the gene *GPM1* was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 37 and SEQ.ID.NO. 38. A restriction-site for Sall, proximal to the DNA fragment and a restriction-site for AvrII, distal to DNA fragment was introduced flanking the intergenic region. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/μl PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 30 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 0.7 % low melt agarose gel and a 547 bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named P-gpm-a8a(+).

Example 14a - TOPO cloning of the *TKL1* gene from the *Saccharomyces cerevisiae* strain D0002 based on NCBI accession code X73224 version 1.

The entire *S. cerevisiae* *TKL1* gene (ScTKL1) was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 39 and SEQ.ID.NO. 40. A restriction-site for XbaI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the stop-codon was introduced flanking the ScTKL1 gene. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/μl PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 90 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 0.7 % low melt agarose gel and a 2059 bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named ScTKL1-a25a(+).

Example 14b - TOPO cloning of the *XKSI* gene from the *Saccharomyces cerevisiae* strain D0002 based on NCBI accession code X61377 version 1.

The entire *S. cerevisiae* *XKS1* gene (ScXKS1) was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 41 and SEQ.ID.NO. 42. A restriction-site for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the stop-codon was introduced flanking the ScXKS1 gene. As template,
5 DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/μl PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 90 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 0.7 % low melt agarose gel and a 2059 bp fragment was isolated. The DNA fragment was
10 TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named ScXKS1-G2.

**Example 14c - TOPO cloning of the *TAL1* gene including 187 bp of transcription terminator from the *Saccharomyces cerevisiae* strain D0002 based on NCBI accession code
15 X15953 version 1.**

The entire *S. cerevisiae* *TAL1* gene together with 187 bp of transcription terminator sequence (ScTAL1+T) was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 43 and SEQ.ID.NO. 44. A restriction-site for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the
20 transcription terminator sequence was introduced flanking the ScXKS1+T gene. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/μl PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 60 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a
25 0.7 % low melt agarose gel and a 1210 bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named ScTAL1+T-a22a(+).

**Example 14d - TOPO cloning of the *RKI1* gene including 206 bp of transcription terminator from the *Saccharomyces cerevisiae* strain D0002 based on NCBI accession code
30 X94335 version 1.**

The entire *S. cerevisiae* *RKI1* gene together with 206 bp of transcription terminator sequence (ScRKI1+T) was PCR amplified from DNA obtained from the *S. cerevisiae* strain D0002 using the primers identified by SEQ.ID.NO. 45 and SEQ.ID.NO. 46. A restriction-site
35 for NheI, proximal to the ATG-start codon and a restriction-site for XhoI, distal to the

transcription terminator sequence was introduced flanking the ScRKI1+T gene. As template, DNA from the *S. cerevisiae* strain was used in a concentration of 0.2 ng/μl PCR-reaction. PCR was performed at 35 cycles of 30 seconds at 96°C, 30 seconds at 57°C, and 60 seconds at 72°C, followed by a final incubation of 10 minutes at 72°C using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland). The PCR product was electrophoretically separated on a 0.7 % low melt agarose gel and a 998 bp fragment was isolated. The DNA fragment was TOPO cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA) according to the manufacturer's instructions and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named ScRKI1+T-a21c(+).

Example 15a - Construction of a plasmid containing the yeast expression cassette composed of the P-pgi promoter in front of the ScXKS1 gene.

The plasmid P-pgi-ala(+) (described in Example 13a) was digested with AvrII and XhoI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid ScXKS1-G2 (described in Example 14b) was digested with NheI and XhoI. The resulting linearized plasmid P-pgi-ala(+) and the DNA fragment containing ScXKS1 originating from the plasmid ScXKS1-G2 were electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid P-pgi-ala(+) was ligated together with the ScXKS1 encoding DNA fragment and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named P-pgi+ScXKS1-b38a.

Example 15b - Construction of a plasmid containing the yeast expression cassette composed of the P-fba promoter in front of the PmXI gene.

The plasmid P-fba-a7b(+) (described in Example 13e) was digested with AvrII and XhoI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid 0717049pGA15 carrying the PmXI gene (described in Example 1b) was digested with NheI and XhoI. The resulting linearized plasmid P-fba-a7b(+) and the DNA fragment containing PmXI originating from the plasmid 0717049pGA15 were electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid P-fba-a7b(+) was ligated together with the PmXI encoding DNA fragment and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named P-fba+PmXI-b34c.

Example 15c - Construction of a plasmid containing the yeast expression cassette composed of the P-gpm promoter in front of the ScRKI1+T gene.

The plasmid P-gpm-a8a(+) (described in Example 13f) was digested with AvrII and XhoI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid ScRKI1+T-a21c(+) (described in Example 13d) was digested with NheI and XhoI. The resulting linearized plasmid P-gpm-a8a(+) and the DNA fragment containing ScRKI1+T

originating from the plasmid ScRKI1+T-a21c(+) were electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid P-gpm-a8a(+) was ligated together with the ScRKI1+T encoding DNA fragment and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named P-gpm+ScRKI1+T-b15a.

5 Example 15d - Construction of a plasmid containing the yeast expression cassette composed of the P-tpi promoter in front of the LIMR gene.

The plasmid P-tpi-a2d(+) (described in Example 13b) was digested with AvrII and XhoI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid 0717050pGA14 (described in Example 1a) was digested with NheI and XhoI. The
 10 resulting linearized plasmid P-tpi-a2d(+) and the DNA fragment containing LIMR originating from the plasmid 0717050pGA14 were electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid P-tpi-a2d(+) was ligated together with the LIMR encoding DNA fragment and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named P-tpi+LIMR-b39a.

15 Example 15e - Construction of a plasmid containing the yeast expression cassette composed of the P-pgm promoter in front of the ScTKL1 gene.

The plasmid P-pgm-a10a(+) (described in Example 13c) was digested with AvrII and XhoI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid ScTKL1-a25a(+) (described in Example 14a) was digested with XbaI and XhoI.
 20 The resulting linearized plasmid P-pgm-a10a(+) and the DNA fragment containing ScTKL1 originating from the plasmid ScTKL1-a25a(+) were electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid P-pgm-a10a(+) was ligated together with the ScTKL1 encoding DNA fragment and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named P-pgm+ScTKL1-b67a.

25 Example 15f - Construction of a plasmid containing the yeast expression cassette composed of the P-pdc promoter in front of the TAL1+T gene.

The plasmid P-pdc-a9f(+) (described in Example 13d) was digested with AvrII and XhoI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid ScTAL1+T-a22a(+) (described in Example 14c) was digested with NheI and XhoI.
 30 The resulting linearized plasmid P-pdc-a9f(+) and the DNA fragment containing ScTAL1+T originating from the plasmid ScTAL1+T-a22a(+) were electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid P-pdc-a9f(+) was ligated together with the ScTAL1+T encoding DNA fragment and the resulting plasmid was used for the transformation of *E. coli* TOP10. The plasmid was named P-pdc+ScTAL1+T-b26a.

Example 16a - Construction of a plasmid with the P-pgi+ScXKS and the P-fba+PmXI concatenated yeast expression cassettes.

The plasmid pgi+ScXKS1-b38a (described in Example 15a) is digested with XhoI and ApaI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly,
5 the plasmid P-fba+PmXI-b34c (described in Example 15b) is digested with SalI and ApaI. The resulting linearized plasmid pgi+ScXKS1-b38a and the DNA fragment containing the P-fba+PmXI expression cassette originating from the plasmid P-fba+PmXI-b34c are electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid pgi+ScXKS1-b38a is ligated together with the DNA fragment containing the
10 P-fba+PmXI expression cassette and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 16b - Construction of a plasmid with the P-tpi+LIMR and the P-pgm+ScTKL1 concatenated yeast expression cassettes.

The plasmid P-tpi+LIMR-b39a (described in Example 15d) is digested with XhoI and
15 ApaI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid P-pgm+ScTKL1-b67a (described in Example 15e) is digested with SalI and ApaI. The resulting linearized plasmid P-tpi+LIMR-b39a and the DNA fragment containing the P-pgm+ScTKL1 expression cassette originating from the plasmid P-pgm+ScTKL1-b67a are electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The
20 linearized plasmid P-tpi+LIMR-b39a is ligated together with the DNA fragment containing the P-pgm+ScTKL1 expression cassette and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 17a - Construction of a plasmid with the P-pgi+ScXKS, the P-fba+PmXI and the P-gpm+ScRKI1+T concatenated yeast expression cassettes.

The plasmid containing the concatenated P-pgi+ScXKS and P-fba+PmXI yeast
25 expression cassettes (described in Example 16a) is digested with XhoI and ApaI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid P-gpm+ScRKI1+T-b15a (described in Example 15c) is digested with SalI and ApaI. The resulting linearized plasmid, containing the concatenated P-pgi+ScXKS and P-fba+PmXI yeast expression cassettes, and the DNA fragment containing the P-gpm+ScRKI1+T expression
30 cassette originating from the plasmid P-gpm+ScRKI1+T-b15a are electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid, containing the concatenated P-pgi+ScXKS and P-fba+PmXI yeast expression cassettes, is ligated together with the DNA fragment containing the P-gpm+ScRKI1+T expression cassette and the resulting
35 plasmid is used for the transformation of *E. coli* TOP10.

Example 17b - Construction of a plasmid with the P-tpi+LIMR, the P-pgm+ScTKL1 and the P-pdc+ScTAL1+T concatenated yeast expression cassettes.

The plasmid containing the concatenated P-tpi+LIMR and P-pgm+ScTKL1 yeast expression cassettes (described in Example 16b) is digested with XhoI and ApaI and resulting
 5 termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid P-pdc+ScTAL1+T-b26a (described in Example 15f) is digested with Sall and ApaI. The resulting linearized plasmid, containing the concatenated P-tpi+LIMR and P-pgm+ScTKL1 yeast expression cassettes, and the DNA fragment containing the P-pdc+ScTAL1+T expression cassette originating from the plasmid P-pdc+ScTAL1+T-b26a are electrophoretically separated
 10 on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid, containing the concatenated P-tpi+LIMR and P-pgm+ScTKL1 yeast expression cassettes, is ligated together with the DNA fragment containing the P-pdc+ScTAL1+T expression cassette and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 17c - Construction of a plasmid with the P-pgm+ScTKL1 and the P-pdc+ScTAL1+T concatenated yeast expression cassettes.

The plasmid P-pgm+ScTKL1-b67a (described in Example 15e) is digested with XhoI and ApaI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Similarly, the plasmid P-pdc+ScTAL1+T-b26a (described in Example 15f) is digested with Sall and ApaI. The resulting linearized plasmid, P-pgm+ScTKL1-b67a, and the DNA fragment
 20 containing the P-pdc+ScTAL1+T expression cassette originating from the plasmid P-pdc+ScTAL1+T-b26a are electrophoretically separated on a 0.7 % low melt agarose gel and thereafter isolated. The linearized plasmid, P-pgm+ScTKL1-b67a is ligated together with the DNA fragment containing the P-pdc+ScTAL1+T expression cassette and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 18a - Construction of a yeast integrative plasmid harbouring the P-pgi+ScXKS, the P-fba+PmXI and the P-gpm+ScRKI1+T concatenated yeast expression cassettes allowing for homologous recombination into the Rdn1 locus and subsequent selection using the antibiotic nourseothricin.

The yeast integrative plasmid allowing for homologous recombination into the Rdn1
 30 locus and subsequent selection on growth media supplemented with nourseothricin (described in Example 12a) is digested with XhoI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Linearized plasmid is electrophoretically separated on a 0.7 % low melt agarose gel, from that of uncut plasmid, and thereafter isolated. Likewise, the plasmid carrying the concatenated P-pgi+ScXKS, P-fba+PmXI and P-gpm+ScRKI1+T yeast expression cassettes
 35 (described in Example 17a) is digested with XhoI and Sall. The concatenated P-pgi+ScXKS, P-

fba+PmXI and P-gpm+ScRKI1+T yeast expression cassette fragment is electrophoretically separated from the vector backbone, using a 0.7 % low melt agarose gel, and thereafter isolated. The linearized integrative plasmid is ligated together with the concatenated P-pgi+ScXKS, P-fba+PmXI and P-gpm+ScRKI1+T yeast expression cassette fragment and the resulting plasmid
 5 is used for the transformation of *E. coli* TOP10.

Example 18b - Construction of a yeast integrative plasmid harbouring the P-tpi+LIMR, the P-pgm+ScTKL1 and the P-pdc+ScTAL1+T concatenated yeast expression cassettes allowing for homologous recombination into the Mig1 locus and subsequent selection using the antibiotic G418.

10 The yeast integrative plasmid allowing for homologous recombination into the Mig1 locus and subsequent selection on growth media supplemented with G418 (described in Example 12b) is digested with XhoI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Linearized plasmid is electrophoretically separated on a 0.7 % low melt agarose gel, from that of uncut plasmid, and thereafter isolated. Likewise, the P-tpi+LIMR, the
 15 P-pgm+ScTKL1 and the P-pdc+ScTAL1+T concatenated yeast expression cassettes (described in Example 17b) is digested with XhoI and Sall. The concatenated P-tpi+LIMR, P-pgm+ScTKL1 and P-pdc+ScTAL1+T yeast expression cassette fragment is electrophoretically separated from the vector backbone, using a 0.7 % low melt agarose gel, and thereafter isolated. The linearized integrative plasmid is ligated together with the concatenated P-tpi+LIMR, P-
 20 pgm+ScTKL1 and P-pdc+ScTAL1+T yeast expression cassette fragment and the resulting plasmid is used for the transformation of *E. coli* TOP10.

**Example 18c - Construction of a yeast integrative plasmid harbouring the P-pgm+ScTKL1 and the P-pdc+ScTAL1+T concatenated yeast expression cassettes allowing for homologous recombination into the Mig1 locus and subsequent selection using the
 25 antibiotic G418.**

The yeast integrative plasmid allowing for homologous recombination into the Mig1 locus and subsequent selection on growth media supplemented with G418 (described in Example 12b) is digested with XhoI and resulting termini subsequently dephosphorylated with alkaline phosphatase. Linearized plasmid is electrophoretically separated on a 0.7 % low melt
 30 agarose gel, from that of uncut plasmid, and thereafter isolated. Likewise, the P-pgm+ScTKL1 and the P-pdc+ScTAL1+T concatenated yeast expression cassettes (described in Example 17c) is digested with XhoI and Sall. The concatenated P-pgm+ScTKL1 and P-pdc+ScTAL1+T yeast expression cassette fragment is electrophoretically separated from the vector backbone, using a 0.7 % low melt agarose gel, and thereafter isolated. The linearized integrative plasmid is ligated

together with the concatenated P-pgm+ScTKL1 and P-pdc+ScTAL1+T yeast expression cassette fragment and the resulting plasmid is used for the transformation of *E. coli* TOP10.

Example 19 - Construction of a *S. cerevisiae* strain containing the P-pgi+ScXKS, the P-fba+PmXI and the P-gpm+ScRKI1+T concatenated yeast expression cassettes by recombination into the Rdn1 locus and subsequent selection using the antibiotic nourseothricin.

Five µg of the integrative plasmid harbouring the P-pgi+ScXKS, the P-fba+PmXI and the P-gpm+ScRKI1+T concatenated yeast expression cassettes (described in Example 18a) is digested with PmeI and subsequently precipitated using 2.5 volumes of 96% ethanol. After discarding the liquid, the DNA pellet is washed with 70 % ethanol, dried and redissolved with 5 µl water. The redissolved digested plasmid is used for the transformation of *S. cerevisiae* yeast strain BY4741 (Euroscarf, Germany) by means of electroporation using the Biorad Gene Pulser II system (Biorad, USA) according to the manufacturer's instruction. Yeast cells are made competent according to a standard protocol (Becker and Guarente 1991). Selection for clones stably transformed with the P-pgi+ScXKS, the P-fba+PmXI and the P-gpm+ScRKI1+T concatenated yeast expression cassettes, are performed by plating on YPD solid growth medium supplemented with 100 mg/L of ClonNAT (Werner BioAgents, Jena, Germany). Prior to plating, the transformed cells are allowed to grow in liquid YPD at 30 °C for 4 hours. After plating on selective media, the plates are incubated at 30 °C for 3 days, a medium size colony is isolated and restreaked on solid YPD supplemented 100 mg/L of ClonNAT and a clone is subsequently isolated.

Example 20a - Construction of a P-pgi+ScXKS, P-fba+PmXI and P-gpm+ScRKI1+T concatenated expression cassette containing *S. cerevisiae* strain including the P-tpi+LIMR, the P-pgm+ScTKL1 and the P-pdc+ScTAL1+T concatenated yeast expression cassettes by recombination into the Mig1 locus and subsequent selection using the antibiotic G418.

Five µg of the integrative plasmid harbouring the P-tpi+LIMR, the P-pgm+ScTKL1 and the P-pdc+ScTAL1+T concatenated yeast expression cassettes (described in Example 18b) is digested with PmeI and subsequently precipitated using 2.5 volumes of 96% ethanol. After discarding the liquid, the DNA pellet is washed with 70 % ethanol, dried and redissolved with 5 µl water. The redissolved digested plasmid is used for the transformation of the *S. cerevisiae* yeast strain described in Example 19 by means of electroporation using the Biorad Gene Pulser II system (Biorad, USA) according to the manufacturer's instruction. Yeast cells are made competent according to a standard protocol (Becker and Guarente 1991). Selection for clones stably transformed with the P-tpi+LIMR, the P-pgm+ScTKL1 and the P-pdc+ScTAL1+T

concatenated yeast expression cassettes, are performed by plating on YPD solid growth medium supplemented with 200 mg/L of geneticin (Life Technologies). Prior to plating, the transformed cells are allowed to grow in liquid YPD at 30 °C for 4 hours. After plating on selective media, the plates are incubated at 30 °C for 3 days, a medium size colony is isolated and restreaked on solid YPD supplemented 200 mg/L of geneticin and a clone is subsequently isolated.

Example 20b - Construction of a P-pgi+ScXKS, P-fba+PmXI and P-gpm+ScRKI1+T concatenated expression cassette containing *S. cerevisiae* strain including the P-pgm+ScTKL1 and the P-pdc+ScTAL1+T concatenated yeast expression cassettes by recombination into the Mig1 locus and subsequent selection using the antibiotic G418.

Five µg of the integrative plasmid harbouring the P-pgm+ScTKL1 and the P-pdc+ScTAL1+T concatenated yeast expression cassettes (described in Example 18c) is digested with PmeI and subsequently precipitated using 2.5 volumes of 96% ethanol. After discarding the liquid, the DNA pellet is washed with 70 % ethanol, dried and redissolved with 5 µl water. The redissolved digested plasmid is used for the transformation of the *S. cerevisiae* yeast strain described in Example 19 by means of electroporation using the Biorad Gene Pulser II system (Biorad, USA) according to the manufacturer's instruction. Yeast cells are made competent according to a standard protocol (Becker and Guarente 1991). Selection for clones stably transformed with the P-pgm+ScTKL1 and the P-pdc+ScTAL1+T concatenated yeast expression cassettes, is performed by plating on YPD solid growth medium supplemented with 200 mg/L of geneticin (Life Technologies). Prior to plating, the transformed cells are allowed to grow in liquid YPD at 30 °C for 4 hours. After plating on selective media, the plates are incubated at 30 °C for 3 days, a medium size colony is isolated and restreaked on solid YPD supplemented 200 mg/L of geneticin and a clone is subsequently isolated.

Example 21a - Measurements of D-xylose metabolism by growth curves of the yeast strains described in Example 20a and 20b.

Changes in the rate of D-xylose metabolism are measured as alterations in the growth rate of the xylose metabolising yeast strains. The two yeast strains (described in Example 20a and 20b) are initially adapted to D-xylose metabolism. Each strain is inoculated individually in liquid Yeast Peptone Xylose (YPX) supplemented with 0.2% glucose. The cultures are incubated for one week at 30°C in a shaker running at 225 RPM. After one week, each culture is used to re-inoculate new cultures with YPX supplemented with 0.02% glucose. The two cultures are incubated for a further week as described above. Growth experiments are initiated by inoculation of YPX at an initial cell titre of OD600 = 0.006/ml. The cultures are incubated as described above. Aliquots are sampled five times with intervals of 24 hours, the optical density, OD600 is measured and growth curves are determined for the two strains. The doubling times

are determined, using the time interval of 24 – 120 hours following the initial lag phase. An increased growth rate of the strain expressing the aldose-1-epimerase (LlMR) together with the xylose isomerase (PmXI), the D-xylulokinase (ScXKS), the ribose-5-phosphate ketol-isomerase (ScRKI1+T), the transketolase (ScTKL1) and the transaldolase (ScTAL1+T) compared to the
5 analogous strain not expressing the mutarotase is demonstrated.

Example 21b - Measurements of D-xylose fermentation of the yeast strains described in Example 20a and 20b.

The two yeast strains adapted to D-xylose metabolism (described in Example 21a) are inoculated individually in liquid Yeast Peptone Xylose (YPX) using baffled shake flasks. The
10 cultures are incubated at 30°C in a shaker running at 225 RPM until OD600 $\approx$ 1.0. Thereafter the cultures are harvested by centrifugation and resuspended in 20 ml of liquid Yeast Peptone supplemented with 50 g/L of D-xylose, using shake flask with two side necks, at a final cell concentration of OD600 = 5. Shake flasks are sealed with bungs containing fermentation locks inserted. Side necks are fitted with gas impermeable resealable rubber stoppers. Fermentation is
15 conducted at 30°C in a shaker running at 100 RPM. Samples are withdrawn, through the resealable rubber stoppers, using a hypodermic needle and syringe and adjusted to pH = 9.0 using 2 M NaOH. Ethanol concentrations are measured using the Megazyme Ethanol Assay Kit: K-ETOH (Megazyme International, Ireland) according to the manufacturer's instruction and with appropriated dilutions of samples. Fermentation progression for each of the two strains is
20 determined by plotting the ethanol concentration as a function of time. Rates are given by the slope of the linear part of the curve, subsequent the initial diauxic shift between aerobic growth and anaerobic fermentation. A faster fermentation rate of the strain expressing the aldose-1-epimerase, compared to the congenetic strain without this gene, is demonstrated.

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The scope of the claims should not be limited by the preferred embodiment and examples, but should be given the broadest interpretation consistent with the description as a whole.

Sequence listing:

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The amino acid sequence encoded by this nucleotide sequence is shown as SEQ ID No 47.

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10 K S I S Q I T L S N E R L T I V

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V H D Y G A R A H Q L L T P D K

145 GTA CAC GAC TAT GGA GCT AGA GCC CAC CAG CTG TTG ACA CCT GAC AAA

15 N G T F E N I L L S K N D S E T

193 AAC GGT ACA TTT GAA AAC ATC TTG TTG TCC AAG AAT GAT TCT GAA ACT

Y A N D G G Y Y G V I C G P V A

20 241 TAT GCA AAT GAT GGC GGC TAT TAT GGT GTT ATT TGT GGT CCT GTT GCT

G R I S G A T Y D S V S L E A N

289 GGC AGA ATA TCT GGA GCT ACT TAT GAC TCA GTG AGC TTA GAA GCC AAC

25 E G K N N L H S G S H G W E R Q

337 GAG GGC AAA AAT AAC TTA CAT TCA GGC TCA CAC GGT TGG GAA AGA CAA

F W S Y E T F E T A S S L G I K

385 TTT TGG AGC TAT GAG ACA TTT GAG ACT GCT TCT TCA TTG GGA ATA AAA

30 L S L R D E E S G F P G Q I Q A

433 CTG TCA TTG AGA GAC GAA GAA TCT GGT TTT CCA GGC CAG ATT CAA GCA

E V T Y K L T D N K L E V T I S

35 481 GAA GTA ACC TAC AAA TTA ACC GAT AAT AAA CTG GAA GTA ACA ATA AGC

G L S V T D T V F N P A W H P Y

529 GGA TTA TCA GTT ACT GAT ACT GTT TTT AAT CCT GCC TGG CAC CCT TAT

80

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 A N V D F L V E T N Q E N I P T
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 R P M S K N R G I A I E F Q E I
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 25 P D L V H H P E W G T I E L K A
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81

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 5 E V M G K K M K D W L R F A M A
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 T K S F P W N E G T D A I E I A
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D A G M L G S I D A N R G D Y Q
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V A M Y Q *

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The amino acid sequence encoded by this nucleotide sequence is shown as SEQ ID No 49.

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D G K P L K E H L R F S V A Y W
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88

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      P  Y  Y  C  F  H  D  V  D  L  V  S  E  G  N  S
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89

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      T   N   F   D   A   K   T   R   R   N   S   T   D   L   E   D
      I   I   I   A   H   V   S   G   M   D   A   M   A   R   A   L
      E   N   A   A   K   L   L   Q   E   S   P   Y   T   K   M   K
10    K   E   R   Y   A   S   F   D   S   G   I   G   K   D   F   E
      D   G   K   L   T   L   E   Q   V   Y   E   Y   G   K   K   N
      G   E   P   K   Q   T   S   G   K   Q   E   L   Y   E   A   I
      V   A   M   Y   Q   *

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15 >SEQ.ID.NO. 49 (ACCESSION NUMBER P22842 version 1):

The amino acid sequence encoded by the nucleotide sequence of
SEQ ID No 3.

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      M   E   Y   F   K   N   V   P   Q   I   K   Y   E   G   P   K
      S   N   N   P   Y   A   F   K   F   Y   N   P   D   E   I   I
20    D   G   K   P   L   K   E   H   L   R   F   S   V   A   Y   W
      H   T   F   T   A   N   G   T   D   P   F   G   A   P   T   M
      Q   R   P   W   D   H   F   T   D   P   M   D   I   A   K   A
      R   V   E   A   A   F   E   L   F   E   K   L   D   V   P   F
      F   C   F   H   D   R   D   I   A   P   E   G   E   T   L   R
25    E   T   N   K   N   L   D   T   I   V   A   M   I   K   D   Y
      L   K   T   S   K   T   K   V   L   W   G   T   A   N   L   F
      S   N   P   R   F   V   H   G   A   A   T   S   C   N   A   D
      V   F   A   Y   A   A   A   Q   V   K   K   A   L   E   I   T
      K   E   L   G   G   Q   N   Y   V   F   W   G   G   R   E   G
30    Y   E   T   L   L   N   T   D   M   E   L   E   L   D   N   L
      A   R   F   L   H   M   A   V   E   Y   A   Q   E   I   G   F
      E   G   Q   F   L   I   E   P   K   P   K   E   P   T   K   H
      Q   Y   D   F   D   A   A   S   V   H   A   F   L   K   K   Y
      D   L   D   K   Y   F   K   L   N   I   E   A   N   H   A   T
35    L   A   G   H   D   F   Q   H   E   L   R   Y   A   R   I   N
      N   M   L   G   S   I   D   A   N   M   G   D   M   L   L   G

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90

W D T D Q Y P T D I R M T T L A
M Y E V I K M G G F N K G G L N
F D A K V R R A S F E P E D L F
L G H I A G M D A F A K G F K V
5 A Y K L V K D G V F D R F I E E
R Y K S Y R E G I G A E I V S G
K A N F K T L E E Y A L N N P K
I E N K S G K Q E L L E S I L N
Q Y L F S E *

CLAIMS

1. A transformed microorganism having: (i) a higher rate of conversion of an aldopentose to a ketopentose than the equivalent microorganism prior to transformation; or (ii) a higher growth rate in the presence of aldopentose than the
5 equivalent microorganism prior to transformation; or (iii) a higher metabolism of aldopentose than the equivalent microorganism prior to transformation;

wherein said microorganism has been transformed with a promoter such that the promoter is operably linked to a nucleotide sequence encoding an aldose-1-epimerase and causes the microorganism to overexpress an aldose-1-epimerase or wherein said
10 microorganism has been transformed with a nucleotide molecule encoding an aldose-1-epimerase; and,

said microorganism has been transformed with at least one expression vector encoding one or more enzymes selected from the group consisting of xylose reductase, D-xylulose reductase, xylose isomerase, arabinose reductase, L-arabitol 4-
15 dehydrogenase, L-xylulose reductase, L-arabinose isomerase, ribulokinase, ribulose phosphate 4-epimerase, D-lyxose isomerase, D-ribose isomerase, xylulokinase, D-ribulokinase, ribose-5-phosphate isomerase, ribulose-5-phosphate epimerase, transaldolase, transketolase and any other enzyme of the pentose phosphate pathway; or

said microorganism has been transformed with a promoter that causes the
20 microorganism to overexpress one or more enzymes selected from the group consisting of xylose reductase, D-xylulose reductase, xylose isomerase, arabinose reductase, L-arabitol 4-dehydrogenase, L-xylulose reductase, L-arabinose isomerase, ribulokinase, ribulose phosphate 4-epimerase, D-lyxose isomerase, D-ribose isomerase, xylulokinase, D-ribulokinase, ribose-5-phosphate isomerase, ribulose-5-phosphate epimerase,
25 transaldolase, transketolase and any other enzyme of the pentose phosphate pathway.

2. The microorganism according to claim 1 wherein said microorganism is a transformed yeast.
- 30 3. The microorganism according to claim 1 or 2 wherein said aldopentose is selected from the group consisting of xylose, arabinose, ribose and lyxose.

4. The microorganism according to any one of claims 1 to 3 wherein said ketopentose is xylulose.
5. An inoculum comprising a microorganism according to any one of claims 1 to 4.
6. A culture medium comprising a microorganism according to any one of claims 1 to 4.
7. A method for preparing a transformed microorganism having: (i) a higher rate of production of a pentose derived compound than the equivalent microorganism prior to transformation; or (ii) a higher rate of conversion of an aldopentose to a ketopentose than the equivalent microorganism prior to transformation; or (iii) a higher growth rate in the presence of aldopentose than the equivalent microorganism prior to transformation; or (iv) a higher metabolism of aldopentose than the equivalent microorganism prior to transformation;
said method comprising the step of transforming a microorganism with a nucleotide molecule encoding an aldose-1-epimerase wherein said microorganism is capable of converting an aldopentose to a pentose derived compound;
wherein said microorganism has been transformed with a promoter such that the promoter is operably linked to a nucleotide sequence encoding an aldose-1-epimerase and causes the microorganism to overexpress an aldose-1-epimerase or wherein said microorganism has been transformed with a nucleotide molecule encoding an aldose-1-epimerase; and,
said microorganism has been transformed with at least one expression vector encoding one or more enzymes selected from the group consisting of xylose reductase, D-xylulose reductase, xylose isomerase, arabinose reductase, L-arabitol 4-dehydrogenase, L-xylulose reductase, L-arabinose isomerase, ribulokinase, ribulose phosphate 4-epimerase, D-lyxose isomerase, D-ribose isomerase, xylulokinase, D-ribulokinase, ribose-5-phosphate isomerase, ribulose-5-phosphate epimerase, transaldolase, transketolase and any other enzyme of the pentose phosphate pathway; or
said microorganism has been transformed with a promoter that causes the microorganism to overexpress one or more enzymes selected from the group consisting of xylose reductase, D-xylulose reductase, xylose isomerase, arabinose reductase, L-

arabitol 4-dehydrogenase, L-xylulose reductase, L-arabinose isomerase, ribulokinase, ribulose phosphate 4-epimerase, D-lyxose isomerase, D-ribose isomerase, xylulokinase, D-ribulokinase, ribose-5-phosphate isomerase, ribulose-5-phosphate epimerase, transaldolase, transketolase and any other enzyme of the pentose phosphate pathway.

5

8. The method according to claim 7 wherein said nucleotide molecule encoding an aldose-1-epimerase is in an expression vector encoding same.

9. A method for producing a pentose derived compound wherein said method
10 comprises culturing in a culture medium a microorganism according to any one of claims 1 to 4.

10. A method for producing a biofuel wherein said method comprises the step of culturing in a culture medium a microorganism according to any one of claims 1 to 4.

15

11. Use of a microorganism for the production of a pentose derived product; wherein said microorganism is:

(a) a transformed microorganism having: (i) a higher rate of conversion of an aldopentose to a ketopentose than the equivalent microorganism prior to
20 transformation; or (ii) a higher growth rate in the presence of aldopentose than the equivalent microorganism prior to transformation; or (iii) a higher metabolism of aldopentose than the equivalent microorganism prior to transformation, wherein said microorganism has been transformed with a promoter such that the promoter is operably linked to a nucleotide sequence encoding an aldose-1-epimerase and causes
25 the microorganism to overexpress an aldose-1-epimerase or wherein said microorganism has been transformed with a nucleotide molecule encoding an aldose-1-epimerase; or

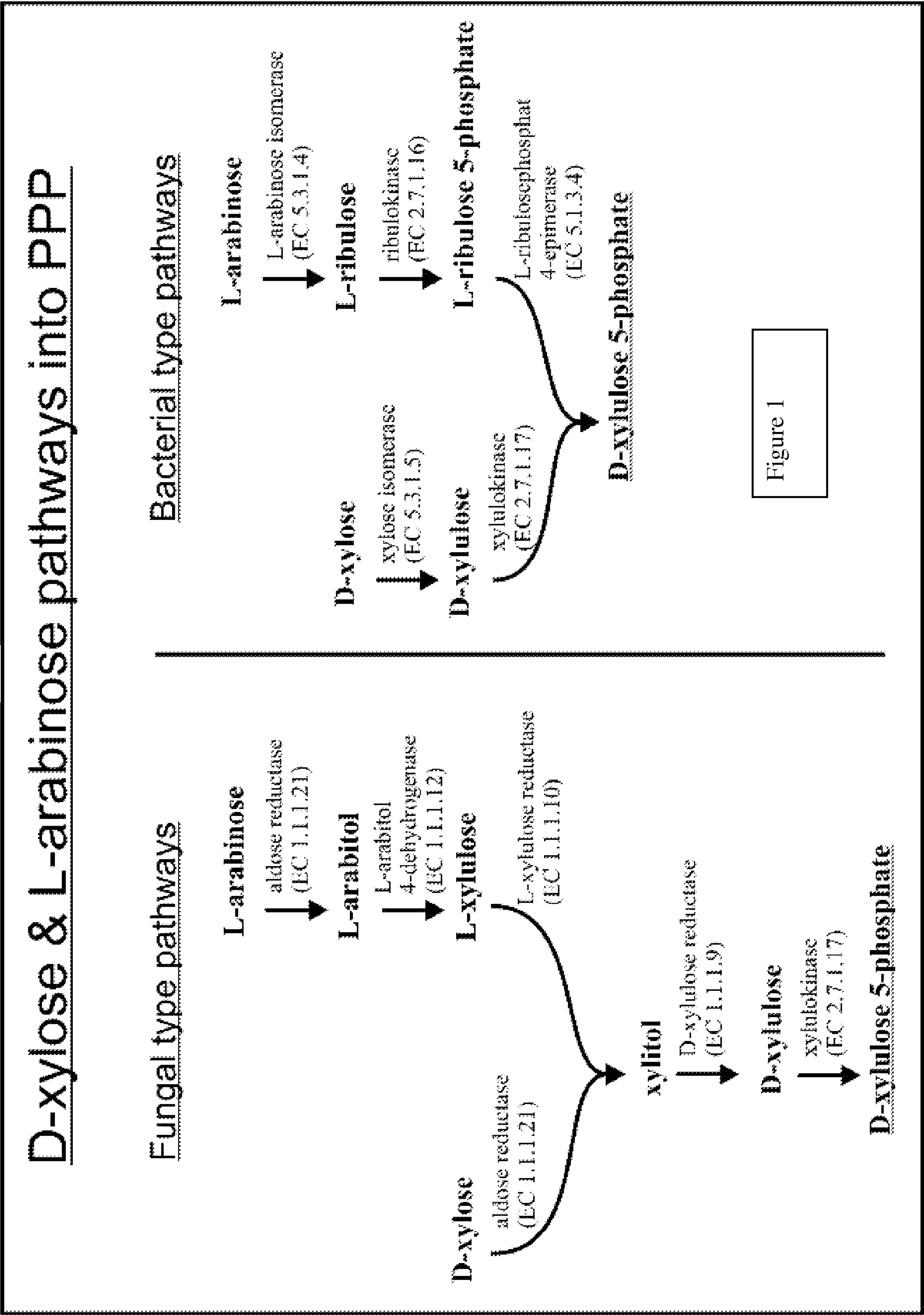
(b) a microorganism according to any one of claims 1 to 4.

30 12. Use of a microorganism for the production of a biofuel; wherein said microorganism is:

(a) a transformed microorganism having: (i) a higher rate of conversion of an aldopentose to a ketopentose than the equivalent microorganism prior to

transformation; or (ii) a higher growth rate in the presence of aldopentose than the equivalent microorganism prior to transformation; or (iii) a higher metabolism of aldopentose than the equivalent microorganism prior to transformation, wherein said microorganism has been transformed with a promoter such that the promoter is
5 operably linked to a nucleotide sequence encoding an aldose-1-epimerase and causes the microorganism to overexpress an aldose-1-epimerase or wherein said microorganism has been transformed with a nucleotide molecule encoding an aldose-1-epimerase; or

(b) a microorganism according to any one of claims 1 to 4.



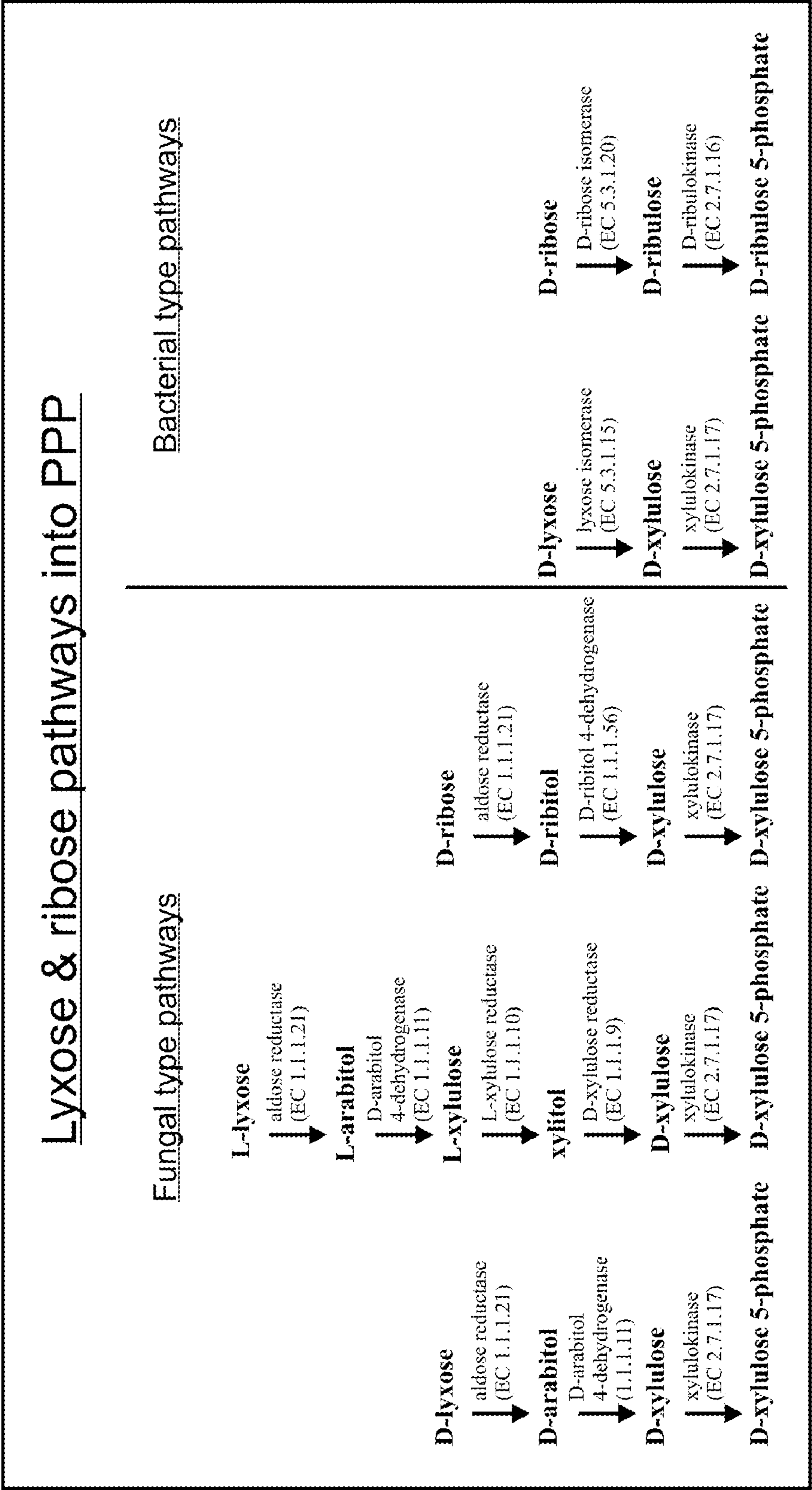


Figure 2

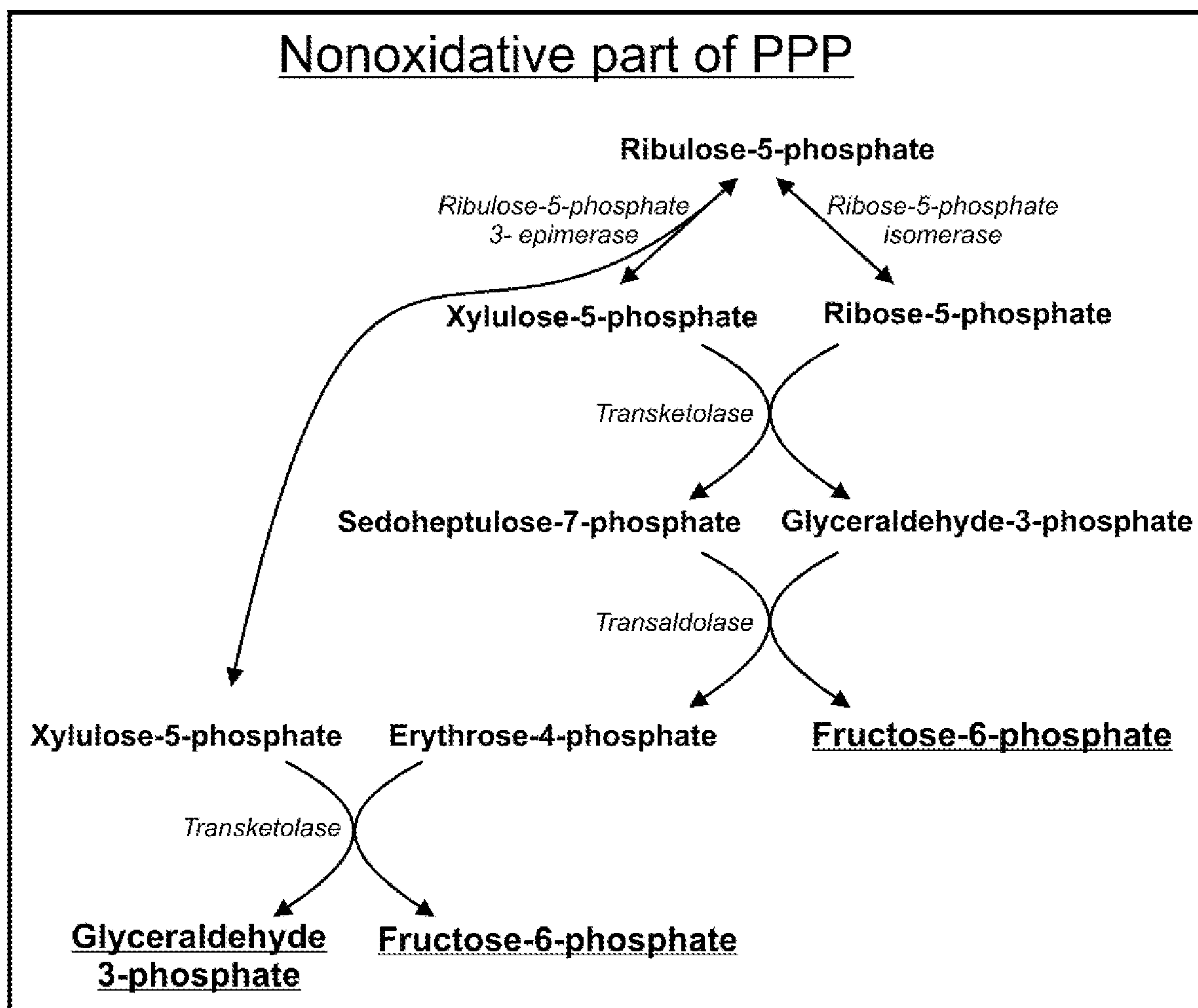


Figure 3

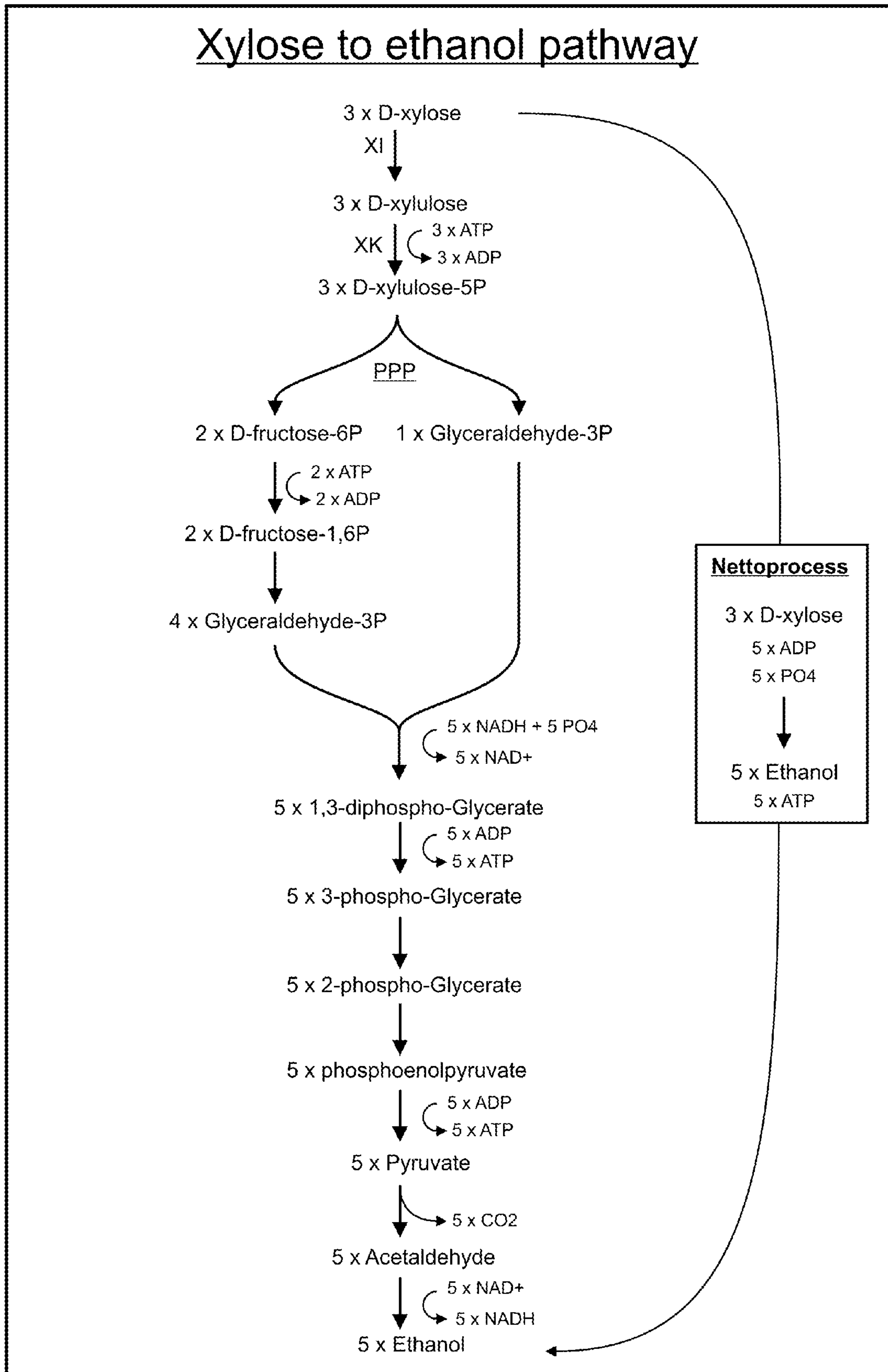


Figure 4