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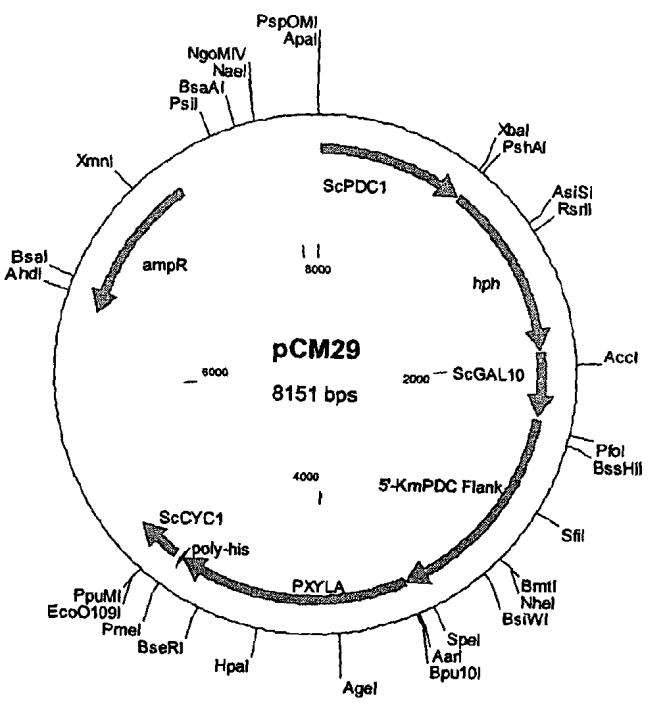
(54) Title: GENETICALLY MODIFIED YEAST SPECIES AND FERMENTATION PROCESSES USING GENETICALLY MODIFIED YEAST



(57) Abstract: Yeast cells are transformed with an exogenous xylose isomerase gene. Additional genetic modifications enhance the ability of the transformed cells to ferment xylose to ethanol or other desired fermentation products. Those modifications', include deletion of non-specific or specific aldose reductase gene(s), deletion of xylitol dehydrogenase gene(s) and/or overexpression of xylulokinase.

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GENETICALLY MODIFIED YEAST SPECIES, AND FERMENTATION PROCESSES USING GENETICALLY MODIFIED YEAST

This invention was made under contract no. DE-FC07-021D14349 with the United States Department of Energy. The United States Government has certain rights to this invention.

This application claims benefit of United States Provisional Application No. 60/467,727, filed May 2, 2003.

This invention relates to certain genetically modified yeast species.

Because of the gradual depletion of world-wide petroleum and natural gas feedstocks, a desire on the part of oil-importing nations to decrease their dependence on foreign sources of oil, and a desire to establish a more sustainable basis to the economy, much effort is being devoted to the production of fuels and organic chemicals and plastics from alternative feedstocks. Fermentation processes offer the possibility of producing a variety of fuels and chemicals from naturally-occurring sugar sources. For example, ethanol is produced in significant quantity by fermenting glucose, most typically glucose obtained by hydrolysing corn starch. A yeast species, *Saccharomyces cerevisiae*, is a common biocatalyst for fermenting glucose to ethanol.

These sugars represent a relatively expensive carbon source. Biomass, i.e. plant matter hydrolysate, offers the possibility of being a particularly inexpensive source of carbon. Biomass consists mainly of cellulose and hemicellulose. Cellulose can be broken down into hexose sugars, typically glucose. Most yeasts, including *S. cerevisiae*, metabolise hexose sugars quite efficiently. Hemicellulose, on the other hand, is rich in pentose sugars such as xylose, so efficient carbon utilization requires that these pentose sugars be metabolised as well. Very few yeast efficiently metabolize xylose to ethanol or other desirable fermentation products. So, in order to exploit the full economic potential offered by using biomass carbon sources, it is necessary to provide a biocatalyst that can efficiently convert xylose to desirable fermentation products.

Various bacteria are capable of metabolising xylose into fermentation products, but these generally produce a mixture of products, rather than a single predominant product as is usually desired. The common by-products are sometimes toxic to the bacteria. Even though certain bacteria have been metabolically engineered to perform homoethanolic fermentions, bacteria tend to perform poorly in

the harsh environment of lignocellulosic hydrolysates, which are a common source of xylose-rich substrates.

Some yeast species such as *S. cerevisiae* are known to ferment hexose sugars predominantly into ethanol, rather than the mixtures of products typically produced by bacteria. Some yeasts have other characteristics that make them good candidates for various types of fermentation process, such as resistance to low pH environments, resistance to certain fermentation co-products such as acetic acid and furfural, and resistance to ethanol itself.

Most yeast species metabolise xylose (if at all) via a complex route, in which xylose is first reduced to xylitol via a xylose reductase (XR) enzyme. The xylitol is then oxidized to xylulose via a xylitol dehydrogenase (XDH) enzyme. The xylulose is then phosphorylated via an XK enzyme. This pathway operates inefficiently in yeast species because it introduces a redox imbalance in the cell. The xylose-to-xylitol step uses NADH as a cofactor, whereas the xylitol-to-xylulose step uses NADPH as a cofactor. Other processes must operate to restore the redox imbalance within the cell. This often means that the organism cannot grow anaerobically on xylose or other pentose sugar.

Nonetheless, attempts have been made to introduce exogenous XR and XDH genes into yeast species such as *S. cerevisiae* in order to achieve conversion of xylose to ethanol. See, for example, US Patent No. 5,866,382, WO 95/13362 and WO 97/42307. The engineered yeast did not produce ethanol efficiently.

Other organisms can isomerise xylose into xylulose and then phosphorylate the xylulose to xylulose 5-phosphate, which then is further metabolised through the cell's central carbon pathway. The isomerization is promoted by a catalytic enzyme, xylose isomerase (XI) and the phosphorylation is catalysed by a xylulokinase (XK) enzyme. This pathway is common in bacteria, but relatively rare in eukaryotic species such as yeast. It does not create the redox imbalance created in the xylose-to-xylitol-to-xylulose pathway, and thus is in principle a more efficient anaerobic mechanism. An anaerobic fungus, *Piromyces* sp. E2 (ATCC 76762), is known to possess a gene that expresses an active XI enzyme.

However, no wild type or recombinant yeast species has had the capacity to efficiently produce desirable fermentation products from xylose or other pentose sugar feedstocks. An attempt to introduce the *Piromyces* sp. E2 XI gene into *S. cerevisiae* resulted in very slow growth on xylose and did not result in reported

ethanol production. *See* Kuyper et al., "High-Level Functional Expression of a Fungal Xylose Isomerase: The Key to Efficient Ethanolic Fermentation of Xylose by Saccharomyces Cerevisiae?", FEMS Yeast Research 1574 (2003) 1-10, and WO 03/062430A1.

A yeast species that can efficiently ferment xylose and other pentose sugars into a desired fermentation product is therefore very desirable.

In one aspect, this invention is a genetically modified yeast cell having a functional, exogenous xylose isomerase gene, wherein the exogenous xylose isomerase gene is operatively linked to promoter and terminator sequences that are functional in the yeast cell, and the modified yeast cell further has a deletion or disruption of a native gene that encodes for an enzyme that catalyzes the conversion of xylose to xylitol.

In a second aspect, this invention is a genetically modified yeast cell of the genera *Kluyveromyces* or *Candida*, having integrated into its genome a functional, exogenous xylose isomerase gene, wherein the exogenous xylose isomerase gene is operatively linked to promoter and terminator sequences that are functional in the yeast cell.

In another aspect, this invention is a genetically modified yeast cell having a functional, exogenous xylose isomerase gene, wherein the exogenous xylose isomerase gene is operatively linked to promoter and terminator sequences that are functional in the yeast cell, and which further contains a functional, exogenous xylulokinase gene operatively linked to promoter and terminator sequences that are functional in the yeast cell.

In still another aspect, this invention is a genetically modified yeast cell having a deletion or disruption of a functional, native gene that produces an enzyme that catalyzes the reaction of xylitol to xylulose or of xylulose to xylitol.

In another aspect, this invention a genetically modified yeast cell having a deletion or disruption of a native gene that produces an enzyme that catalyzes the conversion of xylose to xylitol.

In a still further aspect, this invention is fermentation process in which a cell of any of the preceding aspects is cultured under fermentation conditions in a fermentation broth that includes a pentose sugar.

Figure 1 is a diagram depicting the pNC2 plasmid.

Figure 2 is a diagram depicting the pNC4 plasmid.

Figure 3 is a diagram depicting the pVR22 plasmid.

Figure 4 is a diagram depicting the pVR29 plasmid.

Figure 5 is a diagram depicting the pBH5a and pBH5b plasmids.

Figure 6 is a diagram depicting the pVR78 plasmid assembly from plasmids pVR73 and pVR77.

Figure 7 is a diagram depicting the pCM3 plasmid.

Figure 8 is a diagram depicting the pPS1 plasmid.

Figure 9 is a diagram depicting the pCM9 plasmid.

Figure 10 is a diagram depicting the pCM17 plasmid.

Figure 11 is a diagram depicting the pCM14 plasmid.

Figure 12 is a diagram depicting the pCM28 plasmid.

Figure 13 is a diagram depicting the pVR 95 plasmid.

Figures 14a and 14b are diagrams depicting the pCM18 and pCM19 plasmids.

Figure 15 is a diagram depicting the pBSKura3Km and pBSDeltaUra3KM plasmids.

Figure 16 is a diagram depicting the pVR52, pVR67 and pVR96 plasmids.

Figure 17 is a diagram depicting the pVR102 plasmid.

Figure 18 is a diagram depicting the pVR103 plasmid.

Figure 19 is a diagram depicting the pVR65 and pVR104 plasmids.

Figure 20 is a diagram depicting the pCM21 and pCM23 plasmids.

Figure 21 is a diagram depicting the pCM29 plasmid.

Figure 22 is a diagram depicting the pVR113plasmid.

Figure 23 is a diagram depicting the pCM31 plasmid.

Figure 24 is a diagram depicting the pVR118 plasmid.

Figure 25 is a diagram depicting the pCM52 plasmid.

Figure 26 is a diagram depicting the pCM55 plasmid.

Figure 27 is a diagram depicting the pCM58 plasmid.

Figure 28 is a diagram depicting the pMI409 plasmid.

Figure 30 is a diagram depicting the pMI410 plasmid.

Figure 31 is a diagram depicting the pMI412 plasmid.

Figure 32 is a diagram depicting the pMI403 plasmid.

Figure 33 is a diagram depicting the pMI417 plasmid.

Figure 34 is a diagram depicting the pMI425 plasmid.

Figure 35 is a diagram depicting the pSO91 plasmid.

Figure 36 is a diagram depicting the pSO99 plasmid.

Figure 37 is a diagram depicting the pSO89 plasmid.

Figure 38 is a diagram depicting the pSO96 plasmid.

Figure 39 is a diagram depicting the pSO57 plasmid.

Figure 40 is a diagram depicting the pCM48 plasmid.

The genetically modified yeast of the invention is made by performing certain genetic modifications to a host yeast cell.

A suitable host yeast cell contains at least one native gene that produces an active enzyme that is capable of catalyzing the conversion of D-xylose to xylitol. These may be specific to the xylose→xylitol reduction, or may be non-specific (i.e., operate on a range of pentose sugars). Enzymes produced by such genes are variously referred to by EC number 1.1.1.21, and formally as alditol:NAD(P) 1-oxidoreductase). The enzyme encoded by such genes generally has the following activity: D-xylose + NAD(P)H = xylitol + NAD+ (i.e. it can use either NADPH or NADH as redox cofactors, or both). A gene expressing a xylose reductase enzyme is referred to herein as a "xylose reductase gene", or an "XR gene". In some instances, specific XR genes are designated "XYL1" genes herein.

The term "native" is used herein with respect to genetic materials (e.g., a gene, promoter or terminator) that are found (apart from individual-to-individual mutations which do not affect its function) within the genome of the unmodified cells of that species of yeast.

A host yeast cell capable of converting D-xylose to xylitol will generally have the native ability to further convert xylitol to D-xylulose. This is generally accomplished by expressing a xylitol dehydrogenase (XDH) enzyme that is encoded by a gene referred to herein as a "xylitol dehydrogenase gene" or an "XDH gene". Enzymes encoded by such genes are variously referred to by EC number 1.1.1.9, commonly as xylitol dehydrogenase and systematically a xylitol:NAD+ 2-oxidoreductase (D-xylulose-forming). These genes generally have the following activity: xylitol + NAD(P)+ = D-xylulose + NAD(P)H (although NAD+ is by far the preferred substrate, some do use NADP+). Specific XDH genes are designated "XYL2" genes herein. A suitable host cell has one or more native genes that produce a functional aldose reductase or xylose reductase enzyme and a functional XDH enzyme. An enzyme is "functional" within the context of this invention if it is capable of performing its usual or intended role. A gene is "functional" within the context of this invention if it expresses a functional enzyme.

Another suitable host yeast cell has the ability to transport xylose across its cell wall or membrane.

Another suitable host yeast cell is one that naturally grows on xylose, such as one having an active natural pathway from xylulose-5-phosphate to glyceraldehyde-3-phosphate. In this invention, the pathway from xylulose-5-phosphate to glyceraldehyde-3-phosphate is considered to be active if at least 10% of glucose-based sugars are metabolized by the wild type cell through the hexose monophosphate pathway. Preferably, at least 20, more preferably at least 30%, especially at least 40% of ribulose-5-phosphate is metabolised through this pathway.

Suitable host cells include, for example, yeast cells of the genera *Kluyveromyces, Candida, Pichia, Hansenula, Trichosporon, Brettanomyces, Pachysolen* and *Yamadazyma*. Yeast species of particular interest include *K. marxianus, K. lactis, K. thermotolerans, C. sonorensis, C. methanosorbosa, C. diddensiae, C. parapsilosis, C. naeodendra, C. balnkii, C. entomophila, C. scehatae, P. tannophilus and P. stipitis. K. marxianus, C. sonorensis, C. scehatae, Pachysolen tannophilus* and *Pichia stipitis* are examples of yeast cells that grow on xylose. They have a natural xylulose-5-phosphate to glyceraldehyde-3-phosphate pathway, natural functional aldose and/or xylose reductase genes, active xylitol dehydrogenase genes, and natural ability to transport xylose through the cell wall or membrane. Preferred host cells include those of the species *K. marxianus, K. lactis, K. thermotolerans, C. sonorensis* and *C. methanosorbosa.*

The host cell may contain genetic modifications other than those specifically described herein. For example, the host cell may be genetically modified to produce (or not produce) a particular type of fermentation product by further metabolizing xylulose-5-phosphate and/or glyceraldehyde-3-phosphate. Specific examples of such modifications include the deletion or disruption of a native pyruvate decarboxylase (PDC) gene, and the insertion of exogenous genes such as an L-lactate dehydrogenase (L-LDH) or D-lactate dehydrogenase (D-LDH) gene. Methods for making modifications of these types are described, for example, in WO 99/14335, WO 00/71738, WO 02/42471, WO 03/102201, WO 03/102152 and WO 03/049525. These modifications may be present in the host cell prior to further modifying the host cell as described herein, or may be done simultaneously with or after such further modifications as described herein.

Genetically modified yeast cells of certain aspects of the invention include a functional, exogenous xylose isomerase (XI) gene that is preferably integrated into the genome of the host cell. In this context, "exogenous" means (1) the XI gene is not native to the host cell, (2) the XI gene is native to the host cell, but the genome of the host cell has been modified to provide additional functional copies of the native XI gene, or (3) both (1) and (2). Examples of suitable XI genes include XI genes native to Piromyces species E2 (such as the Piromyces sp. E2 xylA encoding gene sequence in Genbank (Assession # AJ249909)) and *Cyllamyces aberensis* as well as those obtained from other anaerobic fungi. Nucleotide sequences for the Piromyces species E2 *and Cyllamyces Aberensis* XI genes are identified as SEQ. ID. NOs. 58 and 151, respectively. Deduced amino acid sequences for proteins produced by these XI genes are identified as SEQ. ID. No. 59 and 152, respectively. A suitable bacterial XI gene is native to *Bacteroides thetaiotaomicron*. The nucleotide sequence for this *B. thetaiotamicron* XI gene is identified as SEQ. ID. NO. 162. The deduced amino acid sequence for the enzyme produced by this gene is identified as SEQ. ID. NO. 163. Suitable XI genes include those that are at least 60%, 80%, 90%, 95%, 98% or 99% homologous to SEQ. ID. NOs. 58 or 151. Suitable XI genes include those that encode for enzymes that are at least 60%, 80%, 90%, 95%, 98% or 99% homologous to SEQ. ID. NOs. 59 or 152. Some suitable xylose isomerase genes are no greater than 95% or no greater than 90% homologous to SEQ. ID. NO. 58 or encode an enzyme that is no greater than 95% or no greater than 90% homologous to SEQ. ID. NO. 59. Other suitable xylose isomerase genes are bacterial xylose isomerase genes that are at least 60, 80, 90, 95, 98 or 99% homologous to SEQ. ID. NO. 162 and/or produce an enzyme that is at least 60, 80, 90, 95, 98 or 99% homologous to SEQ. ID. NO. 163.

Percent homology of amino acid sequences can conveniently computed using BLAST version 2.2.1 software with default parameters. Sequences having an identities score and a positives score of at least XX%, using the BLAST version 2.2.1 algorithm with default parameters are considered at least XX% homologous. Particularly suitable xylose isomerase genes include those that encode for an enzyme that has an identities score of at least 60%, compared with SEQ. ID. NO. 163, an identities score of less than 95%, compared with SEQ. ID. NO. 59, and a positives score of less than 97%, compared with SEQ. ID. NO. 59.

The exogenous XI gene is under the control of a promoter and a terminator, both of which are functional in the modified yeast cell. As used herein, the term

"promoter" refers to an untranscribed sequence located upstream (*i.e.*, 5') to the translation start codon of a structural gene (generally within about 1 to 1000 bp, preferably 1-500 bp, especially 1-100 bp) and which controls the start of transcription of the structural gene. Similarly, the term "terminator" refers to an untranscribed sequence located downstream (*i.e.*, 3') to the translation finish codon of a structural gene (generally within about 1 to 1000 bp, more typically 1-500 base pairs and especially 1-100 base pairs) and which controls the end of transcription of the structural gene. A promoter or terminator is "operatively linked" to a structural gene if its position in the genome relative to that of the structural gene such that the promoter or terminator, as the case may be, performs its transcriptional control function.

Promoters and terminator sequences may be native to the yeast cell or exogenous. Promoter and terminator sequences that are highly homologous (i.e., 90% or more, especially 95% or more, most preferably 99% or more homologous) in their functional portions to functional portions of promoter and terminator sequences, respectively, that are native to the cell are useful as well, particularly when the insertion of the exogenous gene is targeted at a specific site in the cell's genome.

A suitable promoter is at least 90%, 95% or 99% homologous to a promoter that is native to a yeast gene. A more suitable promoter is at least 90%, 95% or 99% homologous to a promoter for a gene that is native of the host cell. Particularly useful promoters include promoters for yeast pyruvate decarboxylase (PDC), phosphoglycerate kinase (PGK), xylose reductase, (XR), xylitol dehydrogenase (XDH) and transcription enhancer factor-1 (TEF-1) genes, especially from such genes as are native to the host cell.

A suitable terminator is at least 90%, 95% or 99% homologous to a terminator that is native to a yeast gene. The terminator may be at least 90%, 95% or 99% homologous to a terminator for a gene that is native of the host cell. Particularly useful terminators include terminators for yeast pyruvate decarboxylase (PDC), xylose reductase, (XR), xylitol dehydrogenase (XDH) or iso-2-cytochrome c (CYC) genes, or a terminator from the galactose family of genes in yeast, particularly the so-called *GAL10* terminator. A *S. cerevisiae GAL10* terminator and a *S. cerevisiae CYC1* terminator have been shown to be effective terminators for exogenous XI genes in yeast.

The use of native (to the host cell) promoters and terminators, together with respective upstream and downstream flanking regions, can permit the targeted integration of the XI gene into specific loci of the host cell's genome, and for simultaneous integration the XI gene and deletion of another native gene, such as, for example, an XR, XDH or PDC gene.

A poly-his(tidine) tail may be present at the 3' end of the XI gene. A method for accomplishing this is described in Example 3 below. The presence of the poly-his tail may diminish the performance of the XI gene, however. The poly-his tail is not critical to the invention and may be omitted if desired.

The exogenous XI gene may be integrated randomly into the host cell's genome or inserted at one or more targeted locations. Examples of targeted locations include the loci of a gene that is desirably deleted or disrupted, such as an XR, XDH or PDC gene. In some embodiments, integration of the XI gene adjacent to the site of a native PDC gene appears to be related to improved performance of the modified yeast cell in producing fermentation products. Integration at the PDC locus may be accomplished with or without deletion or disruption of the native PDC gene, but it is preferred to maintain the native PDC gene intact and functional, particularly when a desired fermentation product is ethanol or other product that is a pyruvate metabolite.

Targeted integration can be accomplished by designing a vector having regions that are homologous to the upstream (5'-) and downstream (3'-) flanks of the target gene. Either of both of these regions may include a portion of the coding region of the target gene. The XI cassette (including associated promoters and terminators if different from those of the target gene) and selection markers (with associated promoters and terminators as may be needed) will reside on the vector between the regions that are homologous to the upstream and downstream flanks of the target gene.

The genetically modified yeast cell may contain a single copy or multiple copies of the exogenous XI gene. If multiple copies of the exogenous XI gene are present, from 2 to 10 or more copies may be present, such as from about 2-8 or from about 2-5 copies. Multiple copies of the exogenous XI gene may be integrated at a single locus (so they are adjacent each other), or at several loci within the host cell's genome. In an embodiment of particular interest, multiple copies of the exogenous XI gene are incorporated at or adjacent to the locus of a native PDC gene, with or

without deletion or disruption of the native PDC gene. It is possible for different exogenous XI genes to be under the control of different types of promoters and/or terminators.

Performance of the modified yeast, especially under anaerobic conditions, is improved by making one or more additional modifications to its genome, and/or selecting host cells having certain characteristics. These include one or more of (1) low XR (or other aldose reductase) activity, (2) low XDH activity and (3) XK overexpression.

The host cell may naturally have or be modified to have low aldose reductase activity. Such a low aldose reductase activity, measured in the manner described in Example 4E below, is suitably less than 10 mU/mg or less than 5 mU/mg. If the host cell contains one or more aldose reductase genes that produce enzymes that catalyze the conversion of xylose to xylitol, one or more of these genes is suitably disrupted or deleted. In general, the gene(s) selected for disruption or deletion are those which individually or collectively (1) account for at least 40%, preferably at least 50% of the host cell's xylose→xylitol reduction activity, and/or (2) are XR genes, i.e., genes that encode an enzyme specific to the xylose→xylitol reduction. It is generally preferred to delete or disrupt at least one XR gene. Deletion or disruption preferably achieves at least a 50% reduction in enzyme activity, and more preferably reduced xylose reductase activity to below 10 mU/mg or 5 mU/mg.

By "delete or disrupt", it is meant that the entire coding region of the gene is eliminated (deletion), or the gene or its promoter and/or terminator region is modified (such as by deletion, insertion, or mutation) so that the gene no longer produces an active enzyme, or produces an enzyme with severely reduced activity. The deletion or disruption can be accomplished by genetic engineering methods, forced evolution or mutagenesis and/or selection or screening. In the case of the XR or non-specific aldose reductase gene, a suitable method for accomplishing this is to clone the upstream and downstream flanking regions for the gene (which may include a portion of the coding region for the gene), produce a vector containing the cloned upstream and downstream flanks, and transform the host cell with the vector. The vector may contain other genetic material such as a marker gene or other gene that is desirably inserted into the genome of the host cell at the locus of the native XR or non-specific aldose gene (such as an XI gene, XK gene or a gene that enables the cell to produce a desired fermentation product, as an L- or D-LDH gene).

One method of deleting the XR or non-specific aldose reductase gene is to transform the host cell with a vector containing regions that are homologous to the upstream (5'-) and downstream (3'-) flanks of the target gene. Such flanking sequences can be obtained, for example, by amplifying the appropriate regions by PCR using suitably designed primers and genomic DNA as the template. Either of both of these regions may include a portion of the coding region of the target gene, although the vector should not contain the entire functional portion of the gene. Such flanking sequences are generally sequences of at least 50 base pairs, or at least 100 or at least 500 base pairs. Although there is in theory no upper limit to the length of the flanking sequence, it is preferably up to about 4000 base pairs, more preferably up to about 1200 base pairs in length. The flanking sequences are each at least 90%, preferably at least 95%, more preferably at least 98% and even more preferably at least 99% homologous to the corresponding sequences in the cell's genome. These flanking sequences may include the promoter and terminator sequences, respectively, of the target gene. The vector may in addition contain one or more selection marker cassettes (with associated promoters and terminators as may be needed) that advantageously reside between the regions that are homologous to the upstream and downstream flanks of the target gene. Such a vector can delete the target gene in a homologous recombination, inserting the selection marker gene at the locus of the deleted target gene. The vector may instead of or in addition to the selection marker cassette include another expression cassette, such as an XI expression cassette, and L- or D-LDH cassette or a xylulokinase expression cassette, all of which may include associated promoters and terminators. Vectors can also be designed to take advantage of spontaneous loopout events, such as are described in WO 03/102152.

The host cell may naturally have or be modified to have low xylitol dehydrogenase activity. Such a low xylitol dehydrogenase enzyme activity, measured in the manner described in Example 6B below, is suitably less than 2 mU/mg or less than 1 mU/mg. If the host cell contains one or more xylitol dehydrogenase genes resulting in higher xylitol dehydrogenase enzyme activities, one or more of these genes is suitably disrupted or deleted. XDH gene deletion or disruption can be performed in a way analogous to described before with respect to aldose reductase deletion or disruption. Deletion can be performed by incorporating upstream and downstream flanks of the XDH gene into a transformation vector, instead of the flanks of the XR or non-specific aldose reductase gene. As before, the vector may

include one or more selection marker cassettes and/or one or more other expression cassettes. Deletion or disruption preferably achieves at least a 50% reduction in enzyme activity, and more preferably reduced xylitol dehydrogenase activity to below 2 mU/mg or 1 mU/mg.

The modified cell preferably expresses a xylulokinase enzyme having an activity of at least 100 mU/mg, such as at least 300 mU/mg or at least 500 mU/mg, measured as described in Example 5E below. The xylulokinase enzyme is referred to variously as EC 2.7.1.17 and systematically as ATP:D-xylulose 5-phosphotransferase. Its activity is generally ATP + D-xylulose = ADP + D-xylulose 5-phosphateXylulokinase (XK). Overexpression can be achieved, for example, by forced evolution (under conditions that favor selection of mutants that overexpress the enzyme), mutagenesis or by integrating one or more functional exogenous xylulokinase genes into the genome of the host cell. In this context, "exogenous" means (1) the XK gene is not native to the host cell, (2) the XK gene is native to the host cell, but the genome of the host cell has been modified to provide additional functional copies of the native XK gene, or (3) both (1) and (2). Suitable xylulokinase genes include yeast xylulokinase genes. A preferred example of a suitable XK gene is the *S. cerevisiae* XK gene (*ScXKS1*). A nucleotide sequence for the *ScXKS1* gene is identified as SEQ. ID. NO. 83. The deduced amino acid sequence for the enzymes produced by the *ScXKS1* gene is identified as SEQ. ID. NO. 84. Suitable XK genes include those that are at least 70%, 80%, 90%, 95%, 98% or 99% homologous to SEQ. ID. NO. 83. Suitable XK genes include those that encode for enzymes that are at least 70%, 80%, 90%, 95%, 98% or 99% homologous to SEQ. ID. NO. 84. Other suitable XK genes are native to *K. marxianus* or *C. sonorensis*, or are at least 70%, 80%, 80%, 95%, 98% or 99% homologous to either of these.

The exogenous XK gene is under the control of a promoter and a terminator, both of which are functional in the modified yeast cell. Suitable promoters and terminator sequences may be native to the host cell or exhibit a high homology (i.e., 90% or greater, especially 95% or greater, most preferably 99% or greater homology) to a native promoters or terminator. Such promoters and terminators are particularly useful when the exogenous XK gene is targeted at a specific site in the host cell's genome. Other suitable promoters and terminators are native to the organism from which the XK gene was obtained or exhibit a similarly high homology to such native promoter and/or terminators. For example, suitable promoters and

terminators for the *ScXKS1* gene identified above include promoters and terminators for *S. cerevisiae* genes. The promoter and/or terminators may be those native to the particular XK gene or exhibit a similarly high homology to such promoter and/and terminator.

Particularly useful promoters for the *ScXKS1* gene include *S. cerevisiae* pyruvate decarboxylase (PDC), phosphoglycerate kinase (PGK), xylose reductase, (XR), xylitol dehydrogenase (XDH) and transcription enhancer factor-1 (TEF-1) promoters. Particularly useful terminators for the *ScXKS1* gene include *S. cerevisiae* pyruvate decarboxylase (PDC), xylose reductase, (XR), xylitol dehydrogenase (XDH) or iso-2-cytochrome c (CYC) terminators, or a terminator from the galactose family of genes in yeast, particularly the so-called *GAL10* terminator. A *S. cerevisiae* GAL10 terminator and a *S. cerevisiae* CYC1 terminator have been shown to be effective terminators for exogenous XI genes in yeast.

The exogenous XK gene may be integrated randomly into the host cell's genome, or inserted at one or more targeted locations, using methods analogous to those for inserting the XR gene, as discussed above. Examples of targeted locations include the loci of a gene that is desirably deleted or disrupted, such as an XR, XDH or PDC gene. As before, targeted integration can be accomplished by designing a vector having regions that are homologous to the upstream (5'-) and downstream (3'-) flanks of the target gene. Either of both of these regions may include a portion of the coding region of the target gene. The XK cassette (including associated promoters and terminators if different from those of the target gene) and selection markers (with associated promoters and terminators as may be needed) will reside on the vector between the regions that are homologous to the upstream and downstream flanks of the target gene.

The genetically modified yeast cell may contain a single copy or multiple copies (such as from 2 to 10 or more copies, from 2 to 8 or from 2 to 5 copies) of the exogenous XK gene. Multiple copies of the exogenous XK gene may be integrated at a single locus (so they are adjacent each other), or at several loci within the host cell's genome. It is possible for different exogenous XK genes to be under the control of different types of promoters and/or terminators.

Cells according to the invention that have low xylose reductase activity, low xylitol dehydrogenase activity and overexpressed xylulokinase activity are excellent hosts for screening exogenous xylose isomerase genes for activity in the host cell.

These genetic modifications create a cellular environment that tends to favor xylose isomerase expression, so if a certain gene is in fact active, its activity is less likely to be suppressed by the cellular environment and therefore be measurable in the cell.

Genetic modification of the host cell is accomplished in one or more steps via the design and construction of appropriate vectors and transformation of the host cell with those vectors. Electroporation and/or chemical (such as calcium chloride- or lithium acetate-based) transformation methods can be used. Methods for transforming yeast strains are described in WO 99/14335, WO 00/71738, WO 02/42471, WO 03/102201, WO 03/102152 and WO 03/049525; these methods are generally applicable for transforming host cells in accordance with this invention. The DNA used in the transformations can either be cut with particular restriction enzymes or used as circular DNA.

General approaches to transformation vector design have been discussed above in a general sense. Some specific transformation vector designs are as follows, with components listed in order of reading/transcription. All can be circularized or linearized. All may contain restriction sites of various types for linearization or fragmentation. Vectors may further contain a backbone portion (such as for propagation in *E. coli*, which are conveniently obtained from commercially available yeast or bacterial vectors.

1. Upstream (5'-) region of host cell XR gene; marker expression cassette, host downstream (3'-) region of host XR gene. The marker expression cassette may be a hygromycin, Ura3 or G418 resistance expression cassette with promoters and terminators as needed. A Ura3 cassette may be a *HisG-Ura3-HisG* cassette. A G418 cassette may include the *ScPDC1* promoter and *ScGAl10* terminator.

2. Same as (1), with XI cassette (including promoter and terminator operatively linked to the gene) located between the 5'- and 3'- regions of the host cell XR gene. The XI cassette may include a promoter that is native to the host cell. The XI cassette may include a *ScCYC1* or *ScGAL10* terminator.

3. Same as (1) or (2), with XK cassette (including promoter and terminator operatively linked to the gene) located between the 5'- and 3'- regions of the host cell XR gene. The XK cassette may include a promoter that is native to the host cell, or a *ScTEF1* promoter. The XI cassette may include a *ScCYC1* or *ScGAL10* terminator.

4. Upstream (5'-) region of host cell XDH gene; marker expression cassette, host downstream (3'-) region of host XDH gene. The marker expression cassette may be a hygromycin, Ura3 or G418 resistance expression cassette with promoters and terminators as needed. A Ura3 cassette may be a *HisG-Ura3-HisG* cassette. A G418 cassette may include the *ScPDC1* promoter and *ScGAl10* terminator.

5. Same as (4), with XI cassette (including promoter and terminator operatively linked to the gene) located between the 5'- and 3'- regions of the host cell XR gene. The XI cassette may include a promoter that is native to the host cell. The XI cassette may include a *ScCYC1* or *ScGAL10* terminator.

6. Same as (4) or (5), with XK cassette (including promoter and terminator operatively linked to the gene) located between the 5'- and 3'- regions of the host cell XR gene. The XK cassette may include a promoter that is native to the host cell, or a *ScTEF1* promoter. The XI cassette may include a *ScCYC1* or *ScGAL10* terminator.

7. *HisG-Ura3-HisG* cassette preceded or followed by an XI cassette or XK cassette.

8. An XI cassette including a *K. marxianus* promoter or a *C. sonorensis* promoter, the XI cassette being preceded or followed by a marker expression cassette. The *K. marxianus* or *C. sonorensis* promoter may be a PDC or PGK promoter. The terminator in the XI cassette may be a *K. marxianus, C. sonorensis* or *S. cerevisiae* terminator, and may be specifically a *ScCYC1* or *ScGAL10* terminator. The marker expression cassette may be a hygromycin, Ura3 or G418 resistance expression cassette with promoters and terminators as needed. A Ura3 cassette may be a hisG-Ura3-hisG cassette. A G418 cassette may include the *ScPDC1* promoter and *ScGAl10* terminator. The XI cassette may also include an XK cassette (such as described in 9 below), either upstream or downstream of the XI cassette, and either upstream of downstream of the marker expression cassette.

9. An XK cassette being preceded or followed by a marker expression cassette. The XK cassette may include a *K. marxianus* promoter, a *C. sonorensis* promoter or a *S. cerevisiae* promoter. The XK cassette promoter may be specifically a *K. marxianus*, or *C. sonorensis* PDC or PGK or an *S. cerevisiae* PDC, PGC or TEF1 promoter. The terminator in the XK cassette may be a *K. marxianus, C. sonorensis* or *S. cerevisiae* terminator, and may be specifically a *ScCYC1* or *ScGAL10* terminator.

The marker expression cassette may be a hygromycin, Ura3 or G418 resistance expression cassette with promoters and terminators as needed. A Ura3 cassette may be a hisG-Ura3-hisG cassette. A G418 cassette may include the *ScPDC1* promoter and *ScGAl10* terminator.

10. An XI cassette, XK cassette, or both, being preceded by an upstream (5'-) region of a host cell XR gene; and followed by a downstream (3'-) region of a host XR gene. This vector may include other components between the upstream and down stream regions of the XR gene.

11. An XI cassette, XK cassette, or both, being preceded by an upstream (5'-) region of a host cell XDH gene; and followed by a downstream (3'-) region of a host XDH gene. This vector may include other components between the upstream and down stream regions of the XDH gene.

12. Any of the foregoing plasmids further including a self-replication site that is active in the host cell.

Specific XI cassettes useful in the foregoing vectors include the *K. marxianus* PDC1 (*KmPDC1)* promoter, XI gene (any described above), and *ScCYC1, ScGAL10* or *KmPDC1* terminator; the *C. sonorensis PDC1 (CsPDC1)* promoter, XI gene and *ScCYC1, ScGAL10* or *CsPDC1* terminator; and the *C. sonorensis PGK (CsPGK)* promoter, XI gene and *ScCYC1, ScGAL10* or *CsPDC1* terminator.

Specific XK cassettes useful in the foregoing vectors include the *K. marxianus* PDC1 (*KmPDC1)* promoter, XK gene (any described above, but especially the *ScXKS1* gene), and *ScCYC1, ScGAL10* or *KmPDC1* terminator; the *C. sonorensis PDC1 (CsPDC1)* promoter, XK gene and *ScCYC1, ScGAL10* or *CsPDC1* terminator; the *C. sonorensis PGK (CsPGK)* promoter, XK gene and *ScCYC1, ScGAL10* or *CsPDC1* terminator; and *S. cerevisiae* TEF-1 (*ScTEF1*) promoter, XK gene and *ScCYC1, ScGAL10* or *CsPDC1* terminator.

In addition to the specific selection marker genes described above, typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, zeocin (*Streptoalloteichus hindustanus* ble bleomycin resistance gene), G418 (kanamycin-resistance gene of Tn903), hygromycin (aminoglycoside antibiotic resistance gene from *E. coli*), ampicillin, tetracycline, or kanamycin for host cells; (b) complement auxotrophic deficiencies of the cell, such as amino acid leucine deficiency (*K. marxianus* Leu2 gene) or uracil deficiency (*e.g., K. marxianus* or *S. cerevisiae* Ura3 gene); (c) supply critical nutrients not available from simple media, or

(d) confers ability for the cell to grow on a particular carbon source. A xylose isomerase gene can act in this manner, allowing selection to occur on the basis of the ability to grow on xylose.

Successful transformants can be selected for in known manner, by taking advantage of the attributes contributed by the marker gene, or by other characteristics (such as ability to grow on xylose) contributed by the inserted genes. Screening can be performed by PCR or Southern analysis to confirm that the desired insertions and deletions have taken place, to confirm copy number and to identify the point of integration of genes into the host cell's genome. Activity of the enzyme encoded by the inserted gene and/or lack of activity of enzyme encoded by the deleted gene can be confirmed using known assay methods.

The genetically modified yeast cell of the invention containing the exogenous XI gene is useful to ferment pentose sugars to desirable fermentation products such as ethanol and lactic acid. Certain additional genetic modifications may be necessary to enable the yeast cell to produce certain products in acceptable yields, titers and/or productivity For example, integration of an exogenous LDH gene and deletion of native PDC genes may be necessary to obtain high lactic acid yields, as discussed before.

In the fermentation process of the invention, the cell of the invention is cultivated in a fermentation medium that includes a pentose sugar. The pentose sugar is preferably xylose, xylan or other oligomer of xylose. Such sugars are suitably hydrolysates of a hemicelluose-containing biomass. The fermentation medium may contain other sugars as well, notably hexose sugars such as dextrose (glucose) fructose, oligomers of glucose such as maltose, maltotriose and isomaltotriose, and panose. In case of oligomeric sugars, it may be necessary to add enzymes to the fermentation broth in order to digest these to the corresponding monomeric sugar.

The medium will typically contain nutrients as required by the particular cell, including a source of nitrogen (such as amino acids proteins, inorganic nitrogen sources such as ammonia or ammonium salts, and the like), and various vitamins, minerals and the like.

Other fermentation conditions, such as temperature, cell density, selection of substrate(s), selection of nutrients, and the like are not considered to be critical to the invention and are generally selected to provide an economical process. Temperatures during each of the growth phase and the production phase may range from above the

freezing temperature of the medium to about 50°C, although the optimal temperature will depend somewhat on the particular microorganism. A preferred temperature, particularly during the production phase, is from about 30-45°C. When the cell is an engineered *K. marxianus*, it can tolerate relatively high temperatures (such as above 40°C and up to 50°C, especially up to 45°C). Another preferred species of cell, *C. sonorensis*, can tolerate temperatures up to about 40°C. This temperature range provides for the possibility of conducting the fermentation at such higher temperatures (thereby reducing cooling costs) without a significant loss of productivity. Another advantage provided by the good high temperature tolerance is that if the fermentation becomes contaminated with an undesired microorganism, in many cases the undesired microorganism can be selectively killed off by heating the fermentation medium to 40°C or more, especially 45°C or more, without significantly harming the desired cells of the invention.

During the production phase, the concentration of cells in the fermentation medium is typically in the range of about 1-150, preferably about 3-10, even more preferably about 3-6 g dry cells/liter of fermentation medium.

The fermentation may be conducted aerobically, microaerobically or anaerobically. If desired, specific oxygen uptake rate can be used as a process control, as described in WO 03/102200. An advantage of the invention is that the genetically modified cell typically can ferment xylose anaerobically due to the expression of the XI gene and other modifications.

When the fermentation product is an acid, the medium may be buffered during the production phase of the fermentation so that the pH is maintained in a range of about 5.0 to about 9.0, preferably about 5.5 to about 7.0. Suitable buffering agents are basic materials that neutralize lactic acid as it is formed, and include, for example, calcium hydroxide, calcium carbonate, sodium hydroxide, potassium hydroxide, potassium carbonate, sodium carbonate, ammonium carbonate, ammonia, ammonium hydroxide and the like. In general, those buffering agents that have been used in conventional fermentation processes are also suitable here. It is within the scope of the invention, however, to allow the pH of the fermentation medium drop from a starting pH that is typically 6 or higher, to below the pKa of the acid fermentation product, such as in the range of about 2 to about 5 or in the range of from about 2.8 to about 4.5.

In a buffered fermentation, acidic fermentation products such as lactic acid are neutralized as they are formed to the corresponding lactate salt. Recovery of the acid therefore involves regenerating the free acid. This is typically done by removing the cells and acidulating the fermentation broth with a strong acid such as sulfuric acid. A salt by-product is formed (gypsum in the case where a calcium salt is the neutralizing agent and sulfuric acid is the acidulating agent), which is separated from the acid. The acid is then recovered through techniques such as liquid-liquid extraction, distillation, absorption, etc., such as are described in T.B. Vickroy, Vol. 3, Chapter 38 of Comprehensive Biotechnology, (ed. M. Moo-Young), Pergamon, Oxford, 1985; R. Datta, et al., FEMS Microbiol. Rev., 1995; 16:221-231; U.S. Patent Nos. 4,275,234, 4,771,001, 5,132,456, 5,420,304, 5,510,526, 5,641,406, and 5,831,122, and WO 93/00440.

The process of the invention can be conducted continuously, batch-wise, or some combination thereof.

The following examples are provided to illustrate the invention, but are not intended to limit the scope thereof. All parts and percentages are by weight unless otherwise indicated.

Example 1A: Construction of plasmid containing *S. cerevisiae PGK1* promoter and *S. cerevisiae Gal10* terminator (pNC2, Fig. 1); Construction of plasmid containing S. *cerevisiae PDC1* promoter and *S. cerevisiae Gal10* terminator (pNC4, Fig. 2).

The nucleotide sequence of the *S. cerevisiae* PGK1 promoter (*ScPGK1)* is identified as SEQ. ID. NO. 1. This sequence was obtained as a restriction fragment from a proprietary plasmid designated pBFY004. Alternatively, it can be obtained by PCR amplification using *S. cerevisiae* chromosomal DNA as template and primers designed based on SEQ. ID. NO. 1.

The *S. cerevisiae GAL10* terminator (*ScGAL10*) used has the nucleotide sequence identified as SEQ. ID. NO. 2. This sequence was obtained as a restriction fragment from a proprietary plasmid designated pBFY004. Alternatively, it can be obtained by PCR amplification using *S. cerevisiae* chromosomal DNA as template and primers designed based on SEQ. ID NO. 2.

The *S. cerevisiae* PDC1 promoter *(ScPDC1)* was PCR amplified using the primers identified as SEQ ID. NO. 3 and SEQ. ID. NO. 4, using chromosomal DNA from *S. cerevisiae* strain GY5098 (ATCC 4005098) as the template. Thermocycling was performed by 30 cycles of 1 minute at 94°C, 1 minute at 56°C and 1 minute at

72°C, followed by a final incubation of 7 minutes at 72°C, using PfuTurbo DNA polymerase (Stratagene, Madison, WI). The nucleotide sequence of the *ScPDC1* promoter is identified as SEQ. ID. NO. 5.

Plasmid pNC2 (Fig. 1) was generated by combining the *ScPGK1* and the *ScGal10* terminator on the pGEM5Z(+) (Promega, Wisconsin) backbone vector. The *ScPGK1* and the *ScGAL10* were separated in the resulting vector by a poly-linker region with the restriction sites *Xba*I, *Eco*RI and *Bam*HI for inserting particular genes to be expressed between the yeast promoter and terminator. A ~1.2 kbp *NotI* restriction fragment comprised of the *ScPGK1* promoter and *ScGAL10* terminator with multi-cloning sites is identified as SEQ. ID. NO. 6.

The expression vector pNC4 containing expression cassette was constructed in the same general way, except the *ScPDC1* gene was used instead of the *ScPGK1* gene. The resulting vector (pNC4) is shown in Fig. 2. A ~1.3 kbp *NotI* fragment comprised of the *ScPDC1* promoter and *ScGAL10* terminator with multi-cloning sites is identified as SEQ. ID. NO. 7.

Example 1B: Insertion of a G418 resistance marker gene into pNC2 (Ex. 1A, Fig. 1) to create a plasmid in which the G418 gene is operably linked to the *S. cerevisiae PGK1* promoter and *ScGAL10* terminator (pVR22, Fig. 3).

G418 resistance gene was amplified by PCR using *Pfu* Polymerase (Stratagene, Madison, WI) with primers identified as SEQ. ID. NO. 8 and SEQ. ID. NO. 9, using the plasmid pPIC9K (Invitrogen, CA) as the template. Thermocycling was performed by initially incubating the reaction mixture for 5 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 49°C and 2 minutes at 72°C, followed by a final incubation for 10 minutes at 72°C. The PCR product was digested with *Bam*HI and *Xba*I and an 821 bp fragment was isolated and ligated to a ~4303 bp *Bam*HI-*Xba*I fragment of pNC2 (Ex. 1A, Fig. 1). The resulting plasmid (pVR22, Fig. 3) has the *ScPGK1* promoter and *ScGAL10* terminator operably linked to the G418 resistance gene.

Example 1C: Insertion of a G418 resistance marker gene into pNC4 (Ex. 1A, Fig. 2) to create a plasmid in which the G418 gene is operably linked to the *S. cerevisiae PDC1* promoter and *ScGAL10* terminator (pVR29, Fig. 4).

G418 resistance gene was amplified by PCR using Pfu Polymerase (Stratagene, Madison, WI) with primers identified as SEQ. ID. NO. 8 and SEQ. ID. NO. 9, using plasmid pVR22 (Ex. 1B, Fig. 3) as the template. Thermocycling was

performed by initially incubating the reaction mixture for 5 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 49°C, and 2 minutes at 72°C, followed by a final incubation for 10 minutes at 72°C. The PCR product was digested with *Bam*HI and *Xba*I and an 821 bp fragment was isolated and ligated to a ~4303 bp *Bam*HI-*Xba*I fragment of pNC4. (Ex. 1A, Fig. 2). The resulting plasmid, pVR29 (Fig. 4), contained the *ScPDC1* promoter and *ScGAL10* terminator operably linked to the G418 resistance gene.

Example 1D: Construction of a vector (pBH5b, Fig. 5) containing the 5'- and 3'- flanking sequences of the *K. marxianus PDC1* gene, and the G418 gene under control of the *ScPDC1 promoter* and *ScGAL10* terminator

A 1254 bp fragment of DNA immediately upstream of the *K. marxianus PDC1* (*KmPDC1*) gene was PCR amplified with primers identified as SEQ. ID. NO. 10 and SEQ. ID. NO. 11, using the plasmid pSO21 (described in U. S. Published Patent Application 2004/029256A1) as the template. Thermocycling was performed by initially incubating the reaction mixture for 2 minutes at 94°C, then by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1.5 minutes at 72°C, followed by a final incubation of 7 minutes at 72°C. The PCR product was separated on a 0.8% agarose gel and a ~1254 bp product isolated. The PCR product and the pVR29 plasmid (Ex. 1C, Fig. 4) were both digested with *KpnI* and *SbfI* and ligated to produce a ~6315 bp vector designated as pBH5a (Fig. 5). The pBH5a plasmid contained the G418 resistance gene operatively linked to the *ScPDC1* promoter and *ScGAL10* terminator and a ~1240 bp fragment of DNA homologous to DNA immediately upstream of the *KmPDC1* gene.

A 535 bp fragment of DNA immediately downstream of the *KmPDC1* gene was PCR amplified with primers identified by SEQ. ID. NO. 12 and SEQ. ID. NO. 13, using plasmid pSO21 as the template. Thermocycling was performed by initially incubating the reaction mixture for 2 minutes at 94°C, then by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 72°C, followed by a final incubation of 4 minutes at 72°C. The PCR product was separated on a 0.8% agarose gel and a 535 bp product isolated. The PCR product was digested with *Sbf*I and *Mlu*I and the resulting 529 bp fragment was ligated with the *Sbf*I-*Mlu*I fragment of pBH5a to produce plasmid pBH5b (Fig. 5). The pBH5b plasmid contains sequences corresponding to those flanking the *KmPDC1* gene, i.e., a ~1.2 kbp upstream flanking sequence and a ~0.5 kbp of DNA downstream flanking sequence, with a single *SbfI*

site located between them. The pBH5b plasmid also contains the G418 resistance marker operatively linked to the *ScPDC1* promoter and *ScGAL*10 terminator.

Example 1E: Construction of vector containing poly-his tag and *S. cerevisiae* CYC1 terminator (pVR73, Fig. 6); removal of G418 resistance marker gene from pBH5b (Ex. 1D, Fig. 5) to form vector pVR77 (Fig. 6)

Primers identified as SEQ. ID. NO. 14 and SEQ. ID. NO. 15 were designed based on the pYES6CT vector (Invitrogen, CA) for the amplification of bases containing a multiple cloning site, poly-his tag, and *S. cerevisiae* CYC1 terminator (*ScCYC1*). The primers introduced *SbfI* and *BsmBI* sites to the product. PCR conditions were 30 cycles of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 68°C, followed by a final incubation at 68°C for 10 minutes using Platinum Pfx DNA polymerase (Invitrogen, CA). The PCR product was column purified, followed by addition of adenine nucleotides to the 5' ends of TA cloning using Taq DNA polymerase incubated at 72°C for 10 minutes. The 507 bp product was then TA cloned into a TOPOII TA cloning vector (Invitrogen, CA) and designated pVR73 (Fig. 6). The inclusion of the poly-his tag in this vector will cause genes cloned into the unique *SbfI* site to have the his tag fused to the protein expressed from that gene. This tagging of the protein with the poly-his tag allows for relatively quick western blot detection of the protein using Ni-NTA (HRP) conjugate (Quiagen, USA) and for rapid purification of the expressed gene using Ni-chelating resin and columns (Invitrogen, CA).

Plasmid pBH5b (Ex. 1D, Fig. 5) was digested with *Sph*1 and a ~4.7 kbp fragment that retains the *KmPDC1* promoter and terminator was re-ligated to itself to make plasmid pVR77 (Fig. 6). The G418 antibiotic selection marker from pBH5b was thus eliminated.

Example 1F: Construction of a vector pVR78 (Fig. 6) containing the *KmPDC1* upstream flanking region, multi-cloning site, poly-his tag and *ScCYC1* terminator

Plasmid pVR73 (Ex. 1E, Fig. 6) was digested with enzymes *SbfI* and *BsmBI* to release a 504 bp fragment containing the multi-cloning site, poly-his tag and *ScCYC1* terminator. Vector pVR77 was digested using the same enzymes to produce a ~4249 bp fragment containing the *KmPDC1* upstream and downstream flanks and vector backbone. The two fragments were ligated to form a ~4752 bp plasmid (pVR78, Fig. 6) that contained the unique *SbfI* restriction site 184 bp from the poly-his tag. This

process removed most of the *KmPDC1* downstream flanking region from plasmid pVR78.

Example 1G: Modification of plasmid pVR78 (Ex. 1F, Fig. 6) to form plasmid pCM3 (Fig. 7) with a reduced distance from the *SbfI* restriction site to the poly-his tag for better gene expression

Primers identified as SEQ. ID. NO. 16 and SEQ. ID. NO. 17 were designed to amplify the entire region of plasmid pVR78 from the poly-his tag to the *ScCYC1* terminator. The primers also had a 5' S*bf*I site immediately upstream of the poly-his tag and 3' *Sap*I site. PCR reaction was performed using standard methods. PCR conditions consisted of an initial incubation at 94°C for 2 minutes, followed by 10 cycles of 30 seconds at 94°C, 30 seconds at 63°C and 1 minute at 68°C. This was followed by an additional 20 cycles of 30 seconds at 94°C, 1 minute at 68°C. The final step was an 8-minute incubation at 68°C. Amplification was performed using Platinum Pfx DNA polymerase (Invitrogen, CA). The PCR product was digested with *Sbf*I and *Sap*I restriction enzymes. A ~3.9 kb fragment obtained by digestion of plasmid pVR78 with the enzymes 5'*Sbf*I and 3'*Sap*I was ligated to the PCR product. This resulting plasmid was designated pCM3 (Fig. 7).

Example 1H: Construction of a plasmid (pPS1, Fig. 8) containing *E. coli* hygromycin resistance gene under transcriptional control of *ScPDC1* promoter and *ScGAL10* terminator

The *E. coli hph* gene that confers resistance to hygromycin B was PCR amplified using the primers identified by SEQ. ID. NO. 18 and SEQ. ID. No. 19, using the plasmid pRLMex30 (Mach *et al.* 1994, *Curr. Genet. 25,* 567-570) as the template. The *hph* gene can also be obtained using the same primers with *E. coli* chromosomal DNA serving as the template. Thermocycling was performed at 30 cycles of 1 minute at 94°C, 1 minute at 56°C, and 3 minutes at 72°C, followed by a final incubation of 7 minutes at 72°C using PfuTurbo DNA polymerase (Stratagene, Madison, WI). The PCR product was electrophoretically separated on a 0.8% agarose gel and a 1026 bp fragment isolated. The 1026 bp fragment was then digested with *Xba*I and *Bam*HI and ligated into the *Xba*I-*Bam*H1 fragment of pNC4 (Ex. 1A, Fig. 2) containing the *ScPDC1* promoter and the *ScGAL10* terminator to give the plasmid pPS1 (Fig. 8).

Example 1I: Construction of a vector (pCM9, Fig. 9) containing the *KmPDC1* upstream flanking region, multi-cloning site, poly-his tag, *ScCYC1* terminator (all from pCM3, Ex. 1G, Fig. 7) and the *E. coli* hygromycin resistance gene under transcriptional control of *ScPDC1* promoter and *ScGAL10* terminator (from pPS1, Ex. 1H, Fig. 8)

Plasmid pPS1 was digested with *Sph*I and a ~2.2 kbp fragment containing the *hph* gene under the control of the *ScPDC1* promoter and the *ScGAL10* terminator was ligated to *Sph*I-digested pCM3. The resultant plasmid (pCM9, Fig. 9) contains the *KmPDC*1 promoter region followed by a single *Sbf*I site and the *ScCYC1* terminator for future xylose isomerase gene expression. Additionally this cassette for gene expression is positioned right next to a ~2.2kbp fragment containing the *hph* gene under the control of the *ScPDC1* promoter and the *ScGAL10* terminator for selection of the transformants in yeast.

Example 2A: Reconstruction of *Piromyces* sp. E2 xylose isomerase *(PXYLA)* gene based on the sequence available in Genbank

The method used to reconstruct the *Piromyces sp. E2* xylose isomerase *(PXYLA)* gene is adapted from "A method for synthesizing genes and cDNA's by polymerase chain reaction", by Alberto Di Donato et al., Analytical Biochemistry 212, 291-293 (1993). PAGE purified primers are ordered starting from the center of the gene to reconstruct going out. 14 – 16 bp overlaps are maintained for the primer sets. The primers are each 60 – 70 bp long. The gene was reconstructed in 17 steps.

The PCR protocol that was followed during this method used Platinum Pfx (Invitrogen, CA) as the DNA polymerase, and its buffer and $MgSO_4$ as directed by the manufacturer. Step 1 is performed using primers identified as SEQ. ID. NO. 20 and SEQ. ID. NO. 21. These primers represent the center of the gene sequence. No template is required in Step 1, as the annealing of the primers and its extension will form the core template on which subsequent PCR reactions will be built. Cycling for Step 1 is 20 cycles of 94°C for 1 minute, 54°C for 1 minute and 68°C for 2 minutes (with 5 additional seconds being added to each successive cycle), followed by storing at 4°C.

In reaction steps 2-17, Platinum Pfx (Invitrogen, CA) was used as the DNA polymerase, and its buffer and $MgSO_4$ were used as directed by the manufacturer. 2.5 µl of the mix from each step was used as template for each subsequent step (50 µl reaction). The primer sets for each reaction are described in Table 1. The template

in each case was 5 µl of DNA from the preceding reaction step. The cycling for steps 2-17 was 20 cycles of 94°C for 1 minute, 46°C for 1 minute and 68°C for 2 minutes (with 5 additional seconds being added to each successive cycle), followed by storing at 4°C.

Table 1

Reaction Step No.	SEQ. ID. NOs.
1	20, 21
2	22, 23
3	24, 25
4	26, 27
5	28, 29
6	30, 31
7	32, 33
8	34, 35
9	36,37
10	38, 39
11	40, 41
12	42, 43
13	44, 45
14	46, 47
15	48, 49
16	50, 51
17	52,53

Example 2B: Construction of vector pCM17 (Fig. 10) containing the reconstructed *PXYLA* gene; site-directed mutagenesis to alter bases on the reconstructed gene to coincide with the sequence in Genbank database

A plasmid containing the reconstructed *PXYLA* gene (Ex. 2A) was constructed by ligating a ~1.314 kbp fragment produced in the final round of construction to a TOPOII vector (Invitrogen, CA). The reconstructed *PXYLA* gene differed from the Genbank sequence with respect to five bases. Each of these differences was corrected using a multi-site-directed mutagenesis kit (Stratagene, CA), using this plasmid as the template. Three PAGE or HPLC purified, 5' phosphorylated mutagenic primers identified as SEQ. ID. NO. 54, SEQ. ID. NO. 55 and SEQ. ID. NO. 56 were used to correct four of the errors. Thermal cycling parameters included a one-minute denaturation step and a one-minute annealing step, followed by an eight-minute extension. The parental strand formed during the PCR step was then digested by adding 1µl *DpnI* enzyme to the mixture at the end of the thermocycling. The mixture was incubated for 1 hour at 37°C and then used to transform XL10-Gold Ultracompetent *E. coli* cells supplied with the kit. The mixture was plated on Luria-

Bertani + ampicillin (LBA) plates and incubated at 37°C overnight. The multi-site directed mutagenesis protocol was then repeated to fix the fifth error. Two PAGE or HPLC purified, 5'-phosphorylated mutagenic primers identified as SEQ. ID. NO. 57 and SEQ. ID. NO. 55 were used. Two transformants were sequenced and showed 100% homology to the Genbank sequence of the *PXYLA* gene. One of the constructs was named pCM17 (Fig. 10). The nucleotide and amino acid sequences of the reconstructed *PXYLA* gene are identified as SEQ. ID. NO. 58 and SEQ. ID NO. 59.

Example 3A: Construction of vector pCM14 (Fig. 11) containing the *PXYLA* gene under the control of *KmPDC1* promoter and *ScCYC1* terminator, and the *E. coli* hph hygromycin resistance gene under the control of *ScPDC1* promoter and *ScGAL10* terminator

The *PXYLA* gene was PCR amplified using the primers identified as SEQ. ID. NO. 60 and SEQ. ID. NO. 61, using pCM17 (Ex. 2B, Fig. 10) as a template. Thermocycling was performed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 1.5 minutes at 72°C, followed by a final incubation for 8 minutes at 72°C using *Pfx* DNA polymerase (Invitrogen, CA). The PCR product was digested with *Sbf*I and electrophoretically separated on a 1.0% agarose gel. A 1319 bp product was isolated and ligated to a ~6829 bp fragment obtained by digesting pCM9 with *Sbf*I, to construct a ~8148 bp plasmid. A mutagenic primer identified as SEQ. ID. NO. 62 was used to pull out a stop codon which was accidentally added immediately upstream of the *SbfI* site, following the same protocol as described in Example Ex. 2B, to create a ~8148 bp plasmid (pCM14, Fig. 11).

Plasmid pCM14 contains the *PXYLA* gene under the control of the *KmPDC1* promoter and the *ScCYC1* terminator, with a poly-his tail at the 3' end of the gene. The plasmid also contains the *E. coli* hph gene under control of *ScPDC1* promoter and *ScGAL10* terminator.

Example 3B: Incorporation of a *Ura3* selection marker into the pCM14 plasmid (Ex. 3A, Fig. 11), with deletion of the hph expression cassette

Alani et al., in "A method for gene disruption that allows repeated use of *Ura3* selection in the construction of multiply disrupted yeast strains," (Genetics, 1987, 116, 541-545) has described a method of gene integration or gene disruption. This method makes use of a uracil auxotrophic yeast strain and a *HisG-ScUra3-HisG* repeater cassette. This cassette can be used as a selection marker to introduce genes or to disrupt genes with the advantage that the HisG cassette recombines with itself in subsequent generations. Thus the yeast cell loses the *ScUra3* gene during the

recombination event and this loss of *ScUra3* gene restores back the yeast strain's uracil auxotrophy for subsequent transformation with the same cassette.

A *HisG-ScUra3-HisG* cassette was obtained from Nancy DaSilva incorporated in a plasmid designated pNADF11 (Nancy DaSilva, UC Irvine, California). The *HisG-ScUra3-HisG* cassette was isolated from pNADF11 by digesting the plasmid with *Bam*HI and *Eco*RI enzymes and ligating a ~3.8 kbp fragment to a *Bam*HI and *Eco*RI-digested plasmid pPUC19 (New England Biolabs, USA). The resulting plasmid is designated pVR65 (Fig. 19).

Plasmid pCM14 (Ex. 3A, Fig. 11) was digested with *Aat*II/*Sph*I and electrophoretically separated on a 1% gel. A ~5907 bp fragment containing the *PXYLA* cassette was isolated. Plasmid pVR65 was digested with *Aat*II/*Sph*I and electrophoretically separated on a 1% gel. A ~4293 bp *HisG-ScUra3-HisG* fragment was isolated and ligated to a ~5907 bp fragment from plasmid pCM14, with the inserts isolated. The resulting vector was identified as pCM28 (Fig. 12). It contains the *PXYLA* gene (with poly-his tag) under the control of the *KmPDC1* promoter and *ScCYC1* terminator, and the *HisG-Ura3-HisG* cassette. The *E. coli* hph gene and flanking portions that were present in plasmid pCM14 are eliminated from pCM28.

Example 4A: Cloning of the *Kluyveromyces marxianus* xylose reductase (*KmXYL1*) gene and upstream and downstream flanks.

A 410bp fragment of a putative *K. marxianus* xylose reductase (*KmXYL1*) coding region and approximately 500bp of the promoter has been determined by a partial genome sequencing of a similar *K. marxianus* within the Génolevures project (Genomic Exploration of the Hemiascomycetous Yeasts: 12. *Kluyveromyces marxianus* var. *marxianus* Bertrand Llorente, Alain Malpertuy, Gaëlle Blandin, François Artiguenave, Patrick Wincker and Bernard Dujon *FEBS Letters 487(1)* pp. 71-75). Based upon this sequence and some of the known sequences from other yeast xylose reductase consensus, primers were designed to isolate the full *KmXYL1* gene sequence and promoter. A genome walking approach was used to obtain sequences upstream and downstream of a *KmXYL1* gene sequence from a wild type strain of *K. marxianus*. A genome walker kit (BD Biosciences, Paolo Alto, CA) was used for obtaining the sequence of the upstream and downstream flanks. Genomic DNA from *K. marxianus* was digested with restriction enzymes from the genome walker kit (Invitrogen, CA). A genomic library made with the fragments was used as template for PCR reactions.

PCR primers identified as SEQ. ID. NO. 63 and SEQ. ID. NO. 64 were designed to walk both the 5' end and 3' end to get the xylose reductase and upstream/downstream flanking sequence. These primers were used to walk along with primers AP1 and AP2 from the genome walker kit (BD Biosciences, CA). This set of primers amplified a ~2.5 kbp fragment upstream of the gene. The fragment was sequenced to reveal the sequence of DNA from the extreme ends of the upstream region towards the *KmXYL1* gene. Similarly, primers identified as SEQ. ID. NO. 65 and SEQ. ID. NO. 66 were used to walk along with primers AP1 and AP2 from the genome walker kit. This amplified a ~1.8 kbp fragment downstream of the *KmXYL1* gene. The fragment was sequenced to reveal the sequence of DNA from the extreme ends of the downstream region away from xylose reductase.

The sequence information obtained from the genome walking allowed the design of primers identified as SEQ. ID. NO. 67 and SEQ. ID. NO. 68. These primers were used for thermocycling reactions with 500 ng of genomic DNA from *K. marxianus*. Thermocycling was performed by 30 cycles of 1 minute at 94°C, 1 minute at 56°C, 3 minutes at 72°C, followed by a final incubation of 7 minutes at 72°C using Pfx DNA polymerase (Invitrogen, CA). The PCR product was electrophoretically separated on a 0.8% agarose gel. A ~3.5 kbp product was isolated and ligated into the pCRII topo-cloning vector to give plasmid pVR95 (FIG. 13).

Example 4B: Construction of plasmid pCM19 (Fig. 14b) containing the *KmXYL1* upstream and downstream flanks, and the G418 resistance marker gene under the control of *ScPDC1* promoter and *ScGAL10* terminator.

Primers identified as SEQ. ID. NO. 69 and SEQ. ID. NO. 70 were designed to amplify a ~1.3 kbp fragment from plasmid pVR95 (Ex. 4A, Fig 13). This fragment includes the promoter region of the *KmXYL1* gene as well as ~300 bp of the coding region of the gene. These primers were used for thermocycling reactions with 50ng of plasmid DNA from pVR95 (Ex. 4A, Fig. 13). Thermocycling was performed by 30 cycles of 1 minute at 94°C, 1 minute at 53°C, 1 minute at 68°C, and a final incubation of 8 minutes at 68°C using Pfx DNA polymerase (Invitrogen, CA). The PCR product was electrophoretically separated, digested with *Pst*I and ligated to pVR29 (Ex. 1C, Fig. 4) that was also digested with *Pst*I. The plasmid obtained by this method was verified for correct orientation of the *KmXYL1* gene and promoter region into the pVR29 vector. The plasmid is designated pCM18 (Fig. 14a).

Primers identified as SEQ. ID. NO. 71 and SEQ. ID. NO. 72 were designed to amplify a ~1.1 kbp fragment from pVR95. This fragment includes a region

downstream of the *KmXYL1* gene beyond its terminator. These primers were used for thermocycling reactions with 50ng of plasmid DNA from pVR95. Thermocycling was performed by 30 cycles of 1 minute at 94°C, 1 minute at 59°C, 1 minute at 68°C, a final incubation of 8 minutes at 68°C using Pfx DNA polymerase (Invitrogen, CA). Primers identified as SEQ. ID. NO. 73 and SEQ ID. NO. 74 were used for thermocycling reactions with 50 ng of the first PCR product described above to amplify it. Thermocycling was performed by 30 cycles of 1 minute at 94°C, 1 minute at 45°C, 1 minute at 68°C, a final incubation of 8 minutes at 68°C using Pfx DNA polymerase (Invitrogen, CA). The PCR product obtained after the second thermocycling was electrophoretically separated, digested with *Apa*I and ligated to plasmid pCM18 that was also digested with *Apa*I, to form vector pCM19 (Fig. 14b). Plasmid pCM19 contained the downstream flank of *KmXYL1* gene and the upstream flank of *KmXYL1* gene (together with ~300 bp of the coding region of the gene), separated by a cassette containing the G418 gene under the control of the *ScPDC1* promoter and *ScGAL10* terminator. Correct orientation of the *KmXYL1* downstream region with regard to the G418 resistance gene was verified.

Example 4C: Construction of a *K. marxianus* uracil auxotroph (CD 683) by replacing a functional *Ura3* gene with a non-functional gene.

The *K. marxianus Ura3 (KmUra3)* gene was isolated using the genomic DNA as template and primers designed based on Genbank sequence (accession no. AF528508). An 804 bp fragment was cloned into pBluescript vector (Stratagene, Wisconsin) and labelled pBSKura3Myra (Fig. 15).

This plasmid was digested with *Eco*RV and a ~4 kbp fragment that has the *KmUra3* gene with a missing *Eco*RV fragment was isolated and re-ligated to form plasmid pBSDeltaUra3Km (Fig. 15). This plasmid had a non-functional gene *(DeltaUra3).* The plasmid was digested using *Kpn*I and *Not*I and used to transform a wild type strain of *K. marxianus.* The transformants were selected on 5-FOA plates. Colonies that grew on these plates were screened using primers designed in the missing region of the *DeltaUra3* gene. Primers were also designed to isolate the entire gene and those fragments were sequenced to indicate that this new shorter non-functional *DeltaUra3* gene had replaced the actual native *KmUra3* gene in the transformants. The successfully transformed strains were designated CD683. Strain CD683 strain did not grow Sc-Ura plates, indicating that it was a uracil auxotroph.

Example 4D: Generation of a *K. marxianus* mutant (CD804) with deleted xylose reductase (*KmXYL1*) gene by transforming strain CD683 (Ex. 4C) with plasmid pCM19 (Ex. 4B, Fig. 14b)

A ~5.2 kbp fragment containing the upstream and downstream regions of the *KmXYL1* gene with a G418 resistance expression cassette in between was obtained by digesting pCM19 with *Pvu*II. This fragment was used to transform *K. marxianus* strain CD683 using standard electroporation methods. The transformed cells were recovered and plated on 10 g/L yeast extract, 20 g/L yeast peptone, 50 g/L glucose (YPD) + 50 μg/ml G418 plates and incubated at 37°C for 48 – 96 hrs. 96 transformants that grew on YPD + 50 μg/ml G418 plates were replica plated on YPX (10 g/L yeast extract, 20 g/L yeast peptone + xylose 50g/L) + 50 μg/ml G418 plates. 73 out of the 96 transformants failed to grow on YPX + 50 μg/ml G418 plates, confirming their inability to use xylose as a carbon source. This inability indicates that the functional *KmXYL1* gene had been deleted and replaced with the G418 cassette in a homologous recombination. Those transformants that were unable to use xylose as a carbon source were designated CD804.

The absence of a functional *KmXYL1* gene was verified using PCR primers identified as SEQ. ID. NO. 75 and SEQ. ID. NO. 76, which were designed to PCR amplify the center of the *KmXYL1* gene. The presence of the G418 gene was verified by PCR using primers identified as SEQ. ID. NO. 77 and SEQ. ID. NO. 78. The results indicated that the G418 resistance gene fragment was integrated at the locus of the *KmXYL1* gene. A further PCR using a set of primers identified as SEQ. ID. NO. 79 and SEQ. ID. NO. 80 further confirms that the G418 resistance gene fragment replaced the native *KmXYL1* gene. Southern analysis further confirms that the xylose reductase gene was eliminated from strain CD804.

Example 4E: Xylose reductase enzyme activity assay for strains CD683 (Ex. 4C) and CD804 (Ex. 4D)

Separate baffled shake flasks (250ml capacity) were inoculated with strains CD683 (Ex. 4C) and CD804 (Ex. 4D). Flasks were incubated at 35°C with shaking at 250 rpm and grown overnight. The media consisted of 20 g/L yeast extract, 10 g/L peptone, and 100 g/L dextrose. After 18 hours, the cells were spun down at 4000G for 5 minutes, washed with 100mM sodium phosphate buffer and re-suspended in 0.5 ml breaking buffer. The breaking buffer consisted of 100 mM sodium phosphate buffer (pH7.0), 1 mM dithiothreitol (DTT), 40 mg phenylmethyl sulfonyl fluoride (PMSF)

(dissolved in 500 µl DMSO), and 4 Protease inhibitor cocktail tablets (Roche, CA) in a 200ml volume. The cells were lysed mechanically using glass beads (Invitrogen, CA) and centrifuged at 14,000G for 15 minutes. The supernatant was removed and run through a PD-10 desalting column according to the kit protocol (Amersham Bioscience). XR enzyme assay test solution consisted of 100 mM sodium phosphate buffer (pH 7.0), 0.2 mM NADPH, 0.5 mM D-xylose in a total volume of 1ml, to which varying amounts of enzyme solution was added and the absorbance was followed at 340 nm. NADPH usage indicates reductase enzyme activity. A blank solution without xylose was used to determine background NADPH usage.

Total protein was determined using Advanced Protein Assay Reagent (Cysoskeleton #ADV01) with BSA as a standard. The xylose reductase enzyme activity of strain CD683, as indicated by NADPH consumption, was 13.7 mU/mg protein. NAPDH consumption by strain CD804 was consistent with a xylose reductase activity of 4.4 mU/mg protein, rather than the expected activity of zero. This NADPH usage by CD804 is attributed to a non-specific aldose reductase enzyme that is carrying out some conversion of xylose to xylulose. Strains CD683 and CD804 are plated alongside each other on YPX plates. Strain CD804 did not show any growth on those plates at the end of 4 days while strain CD683 grew well on those plates.

Example 5A: Construction of plasmid pVR67 (Fig. 16) containing a cloned *Saccharomyces cerevisiae* xylulokinase (*ScXKS1*) gene

S. cerevisiae cells were obtained from the American Type Culture Collection (ATCC Accession #38626) and grown under standard conditions. Genomic DNA from *S. cerevisiae* was extracted using conventional methodologies. PCR amplification reactions were performed using Pfx polymerase (Invitrogen, CA). Each reaction contained *S. cerevisiae* genomic DNA at a concentration of 500ng, each of 4dNTPs (i.e., each of dATP, dGTP, dCTP and dTTP) at a concentration of 0.2 mM, and each of the amplification primers identified as SEQ. ID. NO. 81 and SEQ. ID. NO. 82 at 1 µM. The cycling was performed by an initial incubation for 10 minutes at 94°C, followed by 35 cycles consisting of 15 seconds at 94°C, 15 seconds at 55°C, and 90 seconds at 68°C. A ~1.8 kbp fragment was gel purified using conventional procedures and cloned into TA cloning vector (Invitrogen, CA). The resultant plasmid (pVR67, Fig. 16) was sequenced to verify the *ScXKS1* gene sequence. The gene exhibits excellent homology to the known sequence in Genbank (Accession # X61377). The

nucleotide sequence of the *ScXKS1* gene is identified as SEQ. ID. NO. 83. The amino acid sequence of the enzyme coded by this gene is identified as SEQ. ID. NO. 84.

Example 5B: Construction of plasmid pVR52 (Fig. 16) containing the *S. cerevisiae TEF1* (*ScTEF1*) promoter and *ScGAL10* terminator.

The *S. cerevisiae TEF1* (*ScTEF1*) promoter was cloned out of pTEFZeo (Invitrogen, CA) vector. Primers identified as SEQ. ID. NO. 85 and SEQ. ID. NO. 86 were used to amplify the *ScTEF1* promoter and insert *Xba*I and *Sst*I restriction sites. The PCR product and plasmid pNC2 (Ex. 1A, Fig. 1) were digested with *Xba*I and *Sst*I enzymes and ligated to obtain plasmid pVR52 (Fig. 16).

Example 5C: Construction of plasmid pVR103 (Fig. 18) containing the *ScXKS1* gene under the control of the *ScTEF1* promoter and *ScGal10* terminator.

Plasmid pVR67 (Ex. 5A, Fig. 16) was digested with *XbaI* and *BamHI*. A ~1.8 kbp fragment containing the *ScXKS1* gene was gel purified. Plasmid pVR52 (Ex. 5B, Fig. 16) was also digested with *XbaI* and *BamHI* and the fragment so obtained was ligated to the ~1.8 kbp fragment from pVR67 to form plasmid vector pVR96 (Fig. 16). This plasmid contains the *ScXKS1* gene under the control of the *ScTEF1* promoter and *ScGal10* terminator. In this vector, the ATG start site of the *ScXKS1* gene is about 130 bp away from the end of the *ScTEF1* promoter. To reduce this distance to about 70-73 bp, primers identified as SEQ. ID. NO. 87 and SEQ. ID. NO. 88 were designed that would amplify the *ScTEF1* promoter from pTEFzeo vector with the correct distance and restriction sites. pTEFzeo (Invitrogen, CA) was used as the template. Thermocycling was performed by 30 cycles of 30 seconds at 94°C, 30 seconds at 57°C, and 30 seconds at 72°C, followed by a final incubation of 4 minutes at 72°C using the Failsafe PCR System (Epicentre, Madison, WI). The PCR product was separated on a 0.8% agarose gel and a 460 bp fragment was isolated. A second PCR was performed to amplify this fragment using the primers identified as SEQ. ID. NO. 89 and SEQ. ID. NO. 90. The PCR product was digested with *Eco*RI and ligated to *Eco*RI-digested plasmid pVR96 (Fig. 16). The resultant plasmid (pVR102, Fig. 17) had two *ScTEF1* promoters – the second one being the promoter driving the *ScXKS1* gene. The distance between the end of the promoter and the ATG of the gene was exactly 73 bp. Plasmid pVR102 was digested with *Sph*I and ligated to *Sph*I-digested pPUC19 (New England Biolabs, USA). The resultant plasmid (pVR103, Fig. 18) was

sequenced to verify the *ScXKS1* gene under the control of a *ScTEF1* promoter and *ScGAL10* terminator.

Example 5D: Construction of plasmid pVR104 (Fig. 19) containing the *ScXKS1* expression cassette (from pVR103, Ex. 5C) alongside a *HisG-ScUra3-HisG* cassette (from pVR65, Ex. 3B).

Plasmid pVR65 (Ex. 3B, Fig. 19) was digested with *Sph*I, and the 5'-phosphate ends of the linearized vector were dephosphorylated using shrimp alkaline phosphatase (Roche Diagnostics, USA) following the manufacturer's protocol.

Plasmid pVR103 (Ex. 5C, Fig. 18) was also digested with *Sph*I and a 3.5 kbp fragment that has the *ScXKS1* gene under the control of the *ScTEF1* promoter and *ScGAL10* terminator was ligated to the linearized pVR65 fragment to obtain plasmid pVR104 (Fig. 19).

Example 5E: Generation of a *K. marxianus* mutant (CD805) that has an over-expressed *ScXKS1* gene activity and deleted xylose reductase gene by transforming strain CD804 (Ex. 4D).

A ~6.8 kbp *Pvu*II fragment from plasmid pVR104 (Ex. 5D, Fig. 19) was used to transform strain CD804 (Ex. 4D), using standard electroporation methods. The transformed cells were recovered in YPD medium, plated after 4 hours on SC-Ura plates (Qbiogene, CA) and incubated at 37°C for 48 – 72 hours. Transformants that grew on SC-Ura plates at the end of 72 hours were re-streaked on fresh SC-Ura plates. Re-streaked transformants were screened using colony PCR.

A single positive colony of the transformed strain was inoculated into 50 ml of YPD medium and incubated overnight at 37°C with 200 rpm shaking. 10 µl of this was plated on 5-FOA plates and incubated overnight at 37°C. Colonies that grew were resistant to 5-FOA and were expected to have lost the *ScUra3* gene due to the recombination of the HisG regions. PCR was performed using primers identified as SEQ. ID. NO. 91 and SEQ. ID. NO. 92 to amplify a 700 bp region to indicate an intact *ScXKS1* gene and ~1 kb of the HisG gene. A second primer set identified as SEQ. ID. NO. 93 and SEQ. ID. NO. 94 were designed to amplify a ~1 kbp product between the *ScXKS1* and end of the gene. These two primer sets confirmed that the *ScXKS1* gene had integrated into the chromosome of the transformants and the *ScUra3* gene had been removed by spontaneous recombination of the HisG region. This strain was labelled CD805 and was tested further for increased xylulokinase protein activity.

Primers identified as SEQ. ID. NO. 91 and SEQ. ID. NO. 93 were designed to amplify a ~2.6 kbp product between the *ScTEF1* promoter and the end of the *ScXKS1* gene. Primers identified as SEQ. ID. NO. 92 and SEQ. ID. NO. 95 were designed to amplify a ~1.7 kbp product between the *ScXKS1* gene and the start of the fragment. These two primer sets confirmed that the *ScXKS1* gene had integrated into the chromosome of strain CD805.

Xylulokinase activity assay: Separate baffled shake flasks (250 ml capacity) were inoculated with strains CD804 (Ex. 4D) and CD805 (Ex. 5E). Flasks were placed at 35°C, shaken at 250rpm, and grown overnight. The media consisted of 20 g/L yeast extract, 10 g/L peptone and 100 g/L dextrose. After 16 hours the cells were spun down at 4000G for 5 minutes, washed with 100 mM sodium phosphate buffer and re-suspended in 0.5 ml breaking buffer. The breaking buffer consisted of 100 mM sodium phosphate buffer (pH7.0), 1 mM DTT, 40mg PMSF (dissolved in 500 µl DMSO), and 4 Protease inhibitor cocktail tablets (Roche) in a 200 ml volume. The cells were lysed mechanically using glass beads (Invitrogen, CA) and centrifuged at 14,000G for 15 minutes. The supernatant was removed and run through a PD-10 desalting column according to the kit protocol (Amersham Bioscience). 10 µl of extract was added to a 30°C equilibrated 80 µl XuK mixture (containing 61 mg $Na_2ATP \cdot 3H_20$, 10.0 ml 0.1M HEPES/KOH (pH 7.5), 1.2 ml 0.1M $MgCl_2$ (diluted to 16.0 ml), and 10 µl of 20 mM xylulose for a total volume of 100 ml. Water substituted xylulose as a blank. Reactions were terminated by boiling for two minutes and transferred to ice. 900 µl of Hek2 (40mM HEPES/KOH (pH 7.5), 10 mM $MgCl_2$, 2.5 mM PEP and 0.2 mM NADH) was added and centrifuged at 14,000G for 10 minutes. The supernatant was transferred to a spectrophotometer curvette, and the initial 340 nm baseline absorbance was established. 10 µl of a 1:1:1 mixture of myokinase, pyruvate kinase, and lactate dehydrogenase was added and final absorbance was measured. Total protein was determined using Advanced Protein Assay Reagent (Cysoskeleton #ADV01) with BSA as a standard. The xylulokinase activity measurement for strain CD804 was 69.7 +/- 8.0 mU/mg, while that for strain CD805 was 400.8 +/- 102.7 mU/mg, indicating that CD805 had over-expressed *ScXKS1* activity.

Example 6A: Construction of plasmid pCM23 (Fig. 20) containing upstream and downstream flanks of a *K. marxianus* xylitol dehydrogenase (*KmXYL2*) gene, and a *E. coli* hph gene under the control of a *ScPDC1* promoter and *ScGAL10* terminator

A 988 bp fragment containing the promoter region of *K. marxianus* xylitol dehydrogenase (*KmXYL2*) gene was PCR amplified out of the genome with primers indicated as SEQ. ID. NO. 96 and SEQ. ID. NO. 97. Thermocycling was performed by 35 cycles of 30 seconds at 94°C, 30 seconds at 52°C, 1 min at 68°C, followed by a final incubation for 8 minutes at 68°C using *Pfx* DNA polymerase (Invitrogen, CA). The product was cloned into a TOPOII vector (Invitrogen, CA). Plasmid pUC19 (New England Biolabs, USA) was digested with *Eco*RI and separated on a 1.0% gel. A 2.686 kbp band was isolated from the pUC19 plasmid and ligated to a fragment liberated from the TOPOII plasmid by digestion with *Eco*RI, creating a ~3.674 kbp plasmid referred to as pCM20.

A fragment containing the terminator sequence and downstream region of *KmXYL2* was PCR amplified out of the genome using three sets of primers. The first set of primers is identified as SEQ. ID. NO. 98 and SEQ. ID. NO. 99, which amplified the downstream region of interest. Thermocycling was performed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1 minute at 68°C, followed by a final incubation for 8 minutes at 68°C using *Pfx* DNA polymerase (Invitrogen, CA). The second set of primers is identified as SEQ. ID. NO. 100 and SEQ. ID. NO. 101. These were used to introduce *SphI* sites on both ends using 2.5 µl of the first PCR product as a DNA template. Thermocycling was performed by 35 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 68°C, followed by a final incubation for 8 minutes at 68°C using *Pfx* DNA polymerase. The third set of primers is identified as SEQ. ID. NO. 102 and SEQ. ID. NO. 103. These amplified the previous product using 2.5 µl of the second PCR. Thermocycling was performed by 35 cycles of 30 seconds at 94°C, 30 seconds at 47°C and 1 minute at 68°C, followed by a final incubation for 8 minutes at 68°C using *Pfx* DNA polymerase. The final product was cloned into a TOPOII vector (Invitrogen, CA) and digested with *Sph*I and separated on a 1.0% gel. A ~1.008 kbp fragment was isolated and ligated into *Sph*I-digested plasmid pCM20 (from above) to form a ~4.682 kbp plasmid designated pCM21 (Fig. 20).

Plasmid pCM21 was digested with *Sac*I/*Xba*I and separated on a 1.0% gel. A ~4.665 kbp band was isolated and ligated to a ~2.366 kbp band isolated by digesting plasmid pPS1 (Ex. 1H, Fig. 8) with *Sac*I/*Spe*I. The resulting ~7.031 kbp plasmid was

named pCM23 (Fig. 20). It contains upstream and downstream flanks of the *KmXYL2* gene, separated by an *E. coli* hph gene under the transcriptional control of the *ScPDC1* promoter and *ScGAL10* terminator.

Example 6B: Generation of a *K. marxianus* mutant (CD806) from strain CD805 (Ex. 5E) using a fragment from plasmid pCM23 (Ex. 6A, Fig. 20) to delete the xylitol dehydrogenase gene.

A single colony of strain CD805 was transformed with a fragment from plasmid pCM23 using standard electroporation methods. The transformed cells were recovered in YPD medium and plated after 4 hours on YPD + 150µg/ml hygromycin plates and incubated at 37°C for 48 hours. 86 transformants that grew on YPD + 150 µg/ml hygromycin plates after 48 hours were re-streaked on fresh YPD + 150 µg/ml hygromycin plates. The transformants were screened by PCR for the presence of the native xylitol dehydrogenase with primers identified by SEQ. ID. NO. 104 and SEQ. ID. NO. 105. Thermocycling was performed by an initial cycle of 10 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at 50°C, 1 minute at 72°C, followed by a final incubation for 8 minutes at 72°C using Failsafe enzyme (Epicentre, Wisconsin). A PCR product of 1064 bp indicated an intact xylitol dehydrogenase gene. 15 transformants did not produce the expected product, indicating that that xylitol dehydrogenase gene had been successfully deleted from those 15 transformants.

Those 15 transformants were PCR screened using primers identified as SEQ. ID. NO. 106 and SEQ. ID. NO. 107. This primer set was designed to PCR amplify the 5' ends. A positive result (~1.5 kbp fragment) indicates that the hygromycin resistance gene fragment replaced the *KmXYL2* gene in the transformant's chromosome in a 5' crossover. A third primer set identified as SEQ. ID. NO. 108 and 109 was designed to PCR amplify the 3' ends. A ~1 kbp product indicates that the hygromycin resistance gene fragment replaced the *KmXYL2* gene in the transformant's chromosome in a 3' crossover. Of the 15 transformants, two showed bands corresponding to both PCR products. One of these was labelled strain CD806. Strain CD806 has an over-expressed *ScXKS1* gene activity and deleted xylose dehydrogenase (*KmXYL2*) and xylose reductase (*KmXYL1*) genes.

<u>Xylitol Dehydrogenase activity assay</u>: Separate baffled shake flasks (250 ml capacity) were inoculated with strains CD805 (Ex. 5E) and CD806 (Ex. 6B). Flasks were placed at 33°C, shaken at 250 rpm, and grown overnight. The media consisted of 20 g/L yeast extract, 10 g/L peptone and 100 g/L dextrose. After 16

hours, the cells were spun down at 4000G for 5 minutes, washed with 100 mM sodium phosphate buffer and re-suspended in 0.5ml breaking buffer. The breaking buffer consisted of 100mM sodium phosphate buffer (pH 7.0), 1 mM DTT, 40 mg PMSF (dissolved in 500 µl DMSO) and 4 Protease inhibitor cocktail tablets (Roche) in a 200 ml volume. The cells were lysed mechanically using glass beads (Invitrogen), and centrifuged at 14,000G for 15 minutes. The supernatant was removed and run through a PD-10 desalting column according to the kit protocol (Amersham Bioscience). Sample was added to a test solution consisting of 100 mM sodium phosphate buffer (pH 7.0), 0.2 mM NADH, and 20 mM xylulose. Absorbance was followed at 340 nm. Total protein was determined using Advanced Protein Assay Reagent (Cysoskeleton #ADV01) with BSA as a standard. Enzyme analysis of strain CD805 (Ex. 5E) yielded an enzyme activity of 14.5 +/- 1.6mU/mg, while the activity strain CD806 was 0.0 +/- 0.1mU/mg. These results indicate that xylitol dehydrogenase enzyme activity had been deleted in strain CD806.

Example 7A: Generation of *K. marxianus* mutant (CD882) having exogenous *PXYLA* gene, overexpressed *ScXKS1* gene activity, and deletions of the *KmXYL1* and *KmXYL2* genes by transforming strain CD806 (Ex. 6B) with plasmid pCM28 (Ex. 3B, Fig. 12)

A single colony of strain CD806 was transformed with *NaeI*-digested plasmid pCM28 using standard electroporation methods. The transformants were grown at 37°C overnight, and streaked onto five identical Sc-Ura plates for screening. The presence of the *PXYLA* gene was verified by PCR using primers identified as SEQ. ID. NO. 110 and SEQ. ID. NO. 111. The resulting transformant (designated CD882) contained the reconstructed *PXYLA* gene, overexpressed *ScXKS1* gene activity, and deletions of the *KmXYL1* and *KmXYL2* genes.

Example 7B: Enzymatic and Western Analysis of Strain CD882 (Ex. 7A)

Protein from CD882 was column purified using Probond Ni^{2+} chelating resin (Invitrogen, Carlsbad, CA, USA) binding the 6X poly-his tail. A Ni-NTA-HRP conjugated probe (Qiagen, Valencia, CA, USA) was used for direct detection of tagged proteins. Western analysis of fractions collected during purification further confirms the presence of the XI protein in strain CD882. Enzyme activity measurements were done according to the method described in "The *Streptomyces rubiginosus* xylose isomerase is misfolded when expressed in *Saccharomyces cerevisiae*", Gardonyl et al., 2003. The evaluation confirms xylose isomerase activity in the same fraction where

the 6X poly-his tail *PXYLA* gene had been detected by western analysis in strain CD882.

Example 8A: Construction of plasmid (pCM29, Fig. 21) containing reconstructed *PXYLA* gene with stop codon that prevents encoding of the poly-his tail.

The reconstructed *PXYLA* gene (Ex. 2A) was PCR amplified using the primers identified as SEQ. ID. NO. 112 and SEQ. ID. NO. 113 using pCM17 (Ex. 2B, Fig. 10) as a template. Thermocycling was performed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 1.5 minutes at 72°C, followed by a final incubation for 8 minutes at 72°C using *Pfx* DNA polymerase. The PCR product was digested with *Sbf*I and electrophoretically separated on a 1.0% agarose gel. A 1319 bp product was isolated and ligated to a 6829 bp fragment obtained by digesting plasmid pCM9 (Ex. 1I, Fig. 9) with *Sbf*I to construct a ~8148 bp plasmid (pCM29, Fig. 21). The plasmid contained the *PXYLA* gene with inoperable poly-his tail, under the control of *KmPCD1* promoter and *ScCYC1* terminator, and the *E. coli* hph expression cassette.

Example 8B: Generation of a *K. marxianus* mutant strain (CD861) containing non-his-tagged *PXYLA* gene, overexpressed *ScXKS1* gene activity and deleted xylose dehydrogenase and xylose reductase genes, by transforming strain CD806 (Ex. 6B) with plasmid pCM29 (Ex. 8A, Fig. 21)

A 3.192 Kb *Pvu*II/*Sph*I fragment obtained by digesting pCM29 is used to transform strain CD806, using standard electroporation methods. The transformed cells were recovered in YPD medium and plated after 6 hours on YPX + 300µg/ml G418 + 150µg/ml hygromycin (pH 7.0). After 5 days at 30°C, several hundred small colonies and one larger colony had grown. The large colony was designated CD861.

Genome walking was performed on strain CD861 to ascertain how the *PXYLA* gene had integrated. The *PXYLA* gene was found to have integrated with more than one copy immediately upstream of the promoter region of the native *PDC* gene. One copy was under the control of a ~1236 bp *KmPDC1* promoter region, present in plasmid pCM29, which includes about 142 bp of an upstream gene, and the *ScCYC1* terminator. Another copy was immediately downstream of this *ScCYC1* terminator, and included a 1026 bp promoter that matches the native *KmPDC1* promoter length. This promoter is missing the 142 bp region of the upstream gene and an additional ~68 bp at the 5' end, compared to the promoter in pCM29 and the first copy. The second copy was also under the control of a *ScCYC1* terminator. Immediately

downstream of this second *ScCYC1* terminator was the native, 1026 bp *KmPDC1* promoter, followed by the native *KmPDC1* gene.

Example 8C: Xylose isomerase, xylose reductase, xylitol dehydrogenase and xylulokinase enzyme analysis of CD861 (Ex. 8B)

Separate baffled shake flasks (250 ml capacity) were inoculated with CD806 (Ex. 6B) and CD861 (Ex. 8B). Flasks were incubated at 30°C and grown overnight with shaking at 250 rpm. The media consisted of 20 g/L yeast extract, 10 g/L peptone and 100 g/L dextrose supplemented with 300 µg/ml G418 and 150 µg/ml hygromycin. Cells were lysed using Y-PER solution (Pierce-Rockford, IL) followed by de-salting using PD-10 columns (Amersham Biosciences, Piscataway, NJ). A test solution consisting of 100 mM TES-NaOH, 250 mM xylose, 10 mM $MgCl_2$, 0.6 mM NADH, 1 U SDH (sigma) (pH 7.0) was brought up to 1.0 mL with the addition of 100 µL of cell extract. Xylose isomerase activity was determined using a blank with the same test solution, only without xylose. Xylose isomerase activity for strain CD806 was zero, while that of strain CD861 was 1.07 +/- 0.21U/mg of crude extract. This verifies that strain CD861 contained a functioning xylose isomerase gene.

Xylose reductase, xylitol dehydrogenase and xylulokinase assays were also conducted to verify activity/loss of activity in the final strain. Strain CD861 (Ex. 8B) and strain CD683 (Ex. 4C) were separately grown overnight in media consisting of 20 g/L yeast extract, 10 g/L peptone, and 100 g/L dextrose (supplemented with 300 µg/ml G418 and 150 µg/ml hygromycin for strain CD861). Cells were lysed using the Y-PER solution. Total protein was determined using Advanced Protein Assay Reagent (Cysoskeleton #ADV01) with BSA as a standard. Xylose reductase enzyme activity in strain CD861 was 260 +/- 41.8mU/mL vs. 516.5 +/- 10.6mU/mL for strain CD683. The ~50% reduction in activity indicates the deletion of the xylose reductase gene, with the measured activity being attributed to a non-specific aldose reductase enzyme that is carrying out some conversion of xylose to xylulose. Xylitol dehydrogenase enzyme activity was 12.4 +/- 4.4mU/mL for strain CD861 vs. 110.4 +/- 7.2 mU/mL for strain CD683. Xylulokinase enzyme activity was 370.5 +/-147.6 mU/mL for strain CD861 vs. 44.8 +/- 8.2 mU/mL for strain CD683.

Example 8D: Kinetic analysis of *PXYLA* gene in strain CD861 (Ex. 8B)

A single colony of strain CD861 was inoculated for overnight growth in media consisting of 10 g/L yeast extract, 20 g/L peptone, and 100 g/L dextrose. Cells were harvested by centrifugation, washed once with 10 ml 100 mM sodium phosphate, 1

mM PMSF (pH 7.0) and centrifuged again. To this, 2 ml of Y-PER solution was added and gently re-suspended and the cell solution was incubated at room temperature for 30 minutes with intermittent agitation. Cells were spun down and the supernatant was desalted using PD-10 columns (Amersham Biosciences). Enzyme assays were conducted as described in Example 8C, with the only difference being the substrate was varied from 0.05-10mM xylose. K_m and V_{max} were derived from Michaelis-Menten plots, which gave a corresponding V_{max} of ~1.8 with a corresponding K_m of 2.2 mM xylose. A Linweaver-Burk plot gave a corresponding V_{max} of ~1.0 with a corresponding K_m of 1.2 mM xylose.

Example 8E: Inhibition studies of *PXYLA* activity in strain CD861 (Ex. 8B) using xylitol

Strain CD861 was grown in xylose media containing varying concentrations of xylitol, a known xylose isomerase inhibitor. The K_m for the xylose isomerase enzyme doubled when xylitol levels are increased from 0 to 0.25mM, and doubled again when xylitol levels are increased to 0.50mM.

Example 8F: pH tolerance of *PXYLA* gene in strain CD861 (Ex. 8B)

A single colony of CD861 was inoculated for overnight growth in media consisting of 10 g/L yeast extract, 20 g/L peptone and 100 g/L dextrose. Cells were harvested by centrifugation, washed once with 10 ml 100 mM sodium phosphate, 1mM PMSF (pH 7.0) and centrifuged again. To this, 2 ml of Y-PER solution was added and gently re-suspended and the cell solution was incubated at room temperature for 30 minutes with intermittent agitation. Duplicate test solutions consisted of 100 mM TES-NaOH, 10 mM $MgCl_2$, 0.6 mM NADH, 250 mM Xylose and 1 U SDH, respectively adjusted to pH 7.0, 6.6, 5.9, 4.6 and 3.8 using 5M Lactic Acid (Sigma, USA) in 900 µL volume. To this 100 µL of enzyme or a dilution thereof was added to bring the final volume to 1 mL. Enzyme activity was measured as in Example 8C. The optimum activity was obtained at pH 6.5. At pH 5.9 the protein retained 45% of the maximum activity.

Example 9A: Microaerobic shake flask characterisation of strains CD806 (Ex. 6B), CD861 (Ex. 8B), and CD882 (Ex 7A).

Single colonies of strains CD806, CD861 and CD882 were separately inoculated for overnight growth in 100 ml media consisting of 20 g/L yeast extract, 10 g/L peptone, and 100 g/L dextrose supplemented with 300 µg/ml G418 and 300 µg/ml hygromycin. Cell dry weights were determined and the appropriate volume of media

for 2 grams cell dry weight (gcdw) was centrifuged and re-suspended in 50 ml media consisting of 20 g/L yeast peptone, 10 g/L yeast extract, 50 g/L Xylose and 10 mM $MgCl_2$, at pH 7.0. Cells were placed in a 250 ml baffled shake flask and placed at 30°C with 70 rpm shaking. Strain CD806 produced 0.7 g/L xylitol as its only product. Strain CD882 produced 3.8 g/L xylitol as well as 0.4 g/L acetate as its only measurable products. Strain CD861 produced 13.5 g/L ethanol, 0.4 g/L xylitol and small amounts of glycerol, acetate, and succinic (<2g/L total).

Example 9B: Tube shake flask characterisation of CD806 (Example 6B), CD861 (Example 8B), and CD882 (Example 7A)

Single colonies of strains CD806, CD861 and CD882 were separately inoculated for overnight growth in 100 ml media consisting of 20 g/L yeast extract, 10 g/L peptone, and 100 g/L dextrose supplemented with 300 µg/ml G418 and 300 µg/ml hygromycin. 50ml of overnight growth from each strain was centrifuged, then re-suspended in 25 ml of production media consisting of 20 g/L yeast peptone, 10 g/L yeast extract, 50 g/L xylose and 10 mM $MgCl_2$, at pH 7. A 1 ml aliquot of the cell re-suspension was added to 45 ml of production media in a 50 ml Falcon tube, and initial OD was taken (see appendix for OD values). This was repeated several times to ensure that enough samples could be taken throughout the experiment. The tubes were then placed in production conditions, 33°C and 200 rpm. Strain CD861 produced 21 g/L ethanol and exhibited a volume productivity of 0.3 g/L-hr. under these conditions. Strain CD806 failed to consume any xylose. Strains CD861 and CD882 were both capable of anaerobic grow.

Example 10A: Construction of plasmid pVR113 (Fig. 22) containing *L. helveticus* L-lactate dehydrogenase gene (*LhLDH*) under control of *ScPGK* promoter and *ScGAL10* terminator and *ScUra3* gene without flanking His repeats.

PCR was performed using plasmid pCM28 (Ex. 3B, Fig. 12) as a template and primers identified as SEQ. ID. NO. 114 and SEQ. ID. NO. 115, to introduce *SphI* sites while removing the flanking HisG repeats. Thermocycling was performed by an initial cycle of 10 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 45°C, 1.4 minutes at 72°C, followed by a final incubation for 8 minutes at 72°C using Taq DNA polymerase enzyme (Qiagen, USA). The resulting plasmid was digested with *Sph*I to obtain a ~1.45 kbp fragment containing the *ScUra3* gene without flanking HisG repeats.

A plasmid (identified as pVR39 in Figure 6 and Example 1E of WO 03/102152A1) containing an *L. helveticus* lactate dehydrogenase (*LhLDH*) gene under the control of the *ScPGK* promoter and the *ScGAL10* terminator, and the G418 resistance marker gene under control of the *ScPGK* promoter and *ScGAL10* terminator, was digested with *SphI* to create a ~5.16 kbp fragment. This ~5.16 kbp fragment was ligated to the ~1.45 kbp fragment from above to form a ~6.61 kbp plasmid (pVR113, Fig. 22).

Example 10B: Generation of *K. marxianus* mutant strain (CD896) containing exogenous lactate dehydrogenase gene, non-his-tagged *PXYLA* gene, overexpressed *ScXKS1* gene activity and deleted xylose dehydrogenase and xylose reductase genes, by transforming strain CD861 (Ex. 8B) with plasmid pVR113 (Ex. 10A, Fig. 22)

A single colony of strain CD861 was transformed with plasmid pVR113 using standard electroporation methods. The transformed cells were recovered in YPD for 4 hours followed by plating on Sc-Ura plates. One positive transformant (strain CD896) was selected by PCR screening for the *LhLDH/ScUra3* cassette. The transformant showed positive PCR results with xylose isomerase and xylulokinase primers, and negative for xylose reductase and xylitol dehydrogenase primers as well.

Example 10C: Shake flask characterization of strain CD896 (Ex. 10B)

A single colony of strain CD896 (Ex. 10B) was inoculated into 50 mL of YPD (10 g/L yeast extract, 20 g/L peptone and 100 g/L glucose in a 250 mL baffled shake flask) and grown for 16 hours at 250 rpm and 37°C. From this culture, 3 gcdw was inoculated into YP (10 g/L yeast extract, 20 g/L peptone) with 50 g/L xylose and 23 g/L $CaCO_3$ in shake flasks (microaerobic) and 250 mL glass bottles (anaerobic). The flasks were incubated at 42°C and samples withdrawn for HPLC at random intervals. Fermentation of strain CD896 under microaerobic conditions gave an L-lactic acid titer of 39g/L and yields of 77% - 79% on xylose consumed. The volumetric productivity of L-lactic acid produced was 1 g/L/hr while the initial xylose consumption rate was between 1.0 and 1.4 g/L/hr. Fermentation of strain CD896 on YP + 50 g/L xylose + 23 g/L calcium carbonate under anaerobic production conditions produced 10 g/L L-lactic acid in the first 24 hrs after which xylose consumption stalled.

Example 11A: Construction of duplicate plasmids containing the reconstructed *PXYLA* gene with (pCM31, Fig. 23) and without (pVR118, Fig. 24) encoded poly-his tail, together with the *ScUra3* gene without flanking His repeats.

This experiment was designed to elucidate the effect of the poly-his tag on the activity of the reconstructed *PYXLA* gene. 5 µg of pCM14 (Ex. 3A, Fig. 11) was digested with *Sph*I and electrophoretically separated on a 1.0% agarose gel. A ~5889 bp product was isolated and ligated to a 1450 bp fragment obtained by digesting plasmid pVR113 (Ex. 10A, Fig. 22) with *Sph*I, to form a ~7339 bp plasmid (pCM31, Fig. 23). Plasmid pCM31 contains the *PXYLA* gene with poly-his tail and under control of the *KmPDC1* promoter and *ScCYC1* terminator, and the *ScUra3* expression cassette. Separately, 5 µg of plasmid pCM29 (Ex. 8A, Fig. 21) was digested with *Sph*I, and electrophoretically separated on a 1.0% agarose gel. A ~5892 bp product was isolated, and ligated to a 1450 bp fragment obtained by digesting plasmid pVR113 with *Sph*I to form a ~7342 bp plasmid (pVR118, Fig. 24). Plasmid pVR118 is similar to plasmid pCM31, but contains a stop codon that prevents encoding of the poly-his tail.

Example 11B: Generation of *K. marxianus* mutant strains containing reconstructed *PXYLA* gene with (CD929) and without (CD931) encoded poly-his tag, together with *ScUra3* gene without flanking His repeats, by transforming strain CD806 (Ex. 6B) with plasmids pCM31 (Ex. 11A, Fig. 23) and pVR118 (Ex. 11A, Fig. 24)

A single colony of *K. marxianus* strain CD806 (Ex. 6B) was transformed with plasmid pCM31 using standard electroporation methods. The transformed cells were recovered in YPD medium and plated after 4 hours on Sc-Ura plates. Two transformants were verified by PCR to contain the reconstructed *PXYLA* gene (with encoded poly-his tag) and overexpressed *ScXKS1* gene, and also to confirm the deletions of the *KmXYL1* and *KmXYL2* genes. One of these transformants was designated strain CD929.

K. marxianus strain CD806 was transformed with plasmid pVR118 using standard electroporation methods. A transformant that was verified by PCR to contain the reconstructed *PXYLA* gene (without encoded poly-his tag) and overexpressed *ScXKS1* gene, and to have deletions of the *KmXYL1* and *KmXYL2* genes, was designated strain CD931.

Genome walking of strain CD931 shows that the PXYLA gene had integrated twice, in the same manner as described for strain CD861 (Ex. 8B).

Example 11C: Shake flask characterizations of strains CD929 and CD931 (Ex. 11B)

Single colonies of CD929 and CD931 (Ex. 11B) were separately inoculated into 100 mL of 10 g/L yeast extract, 20 g/L peptone, 70 g/L glucose, 300 µg/ml G418 + 150µg/ml hygromycin in a 250 mL baffled shake flask. The cultures were incubated overnight at 35°C with 250 rpm. The cells were inoculated into 50 mL falcon tubes containing 45 mL production media (10 g/L yeast extract, 20 g/L peptone, 50 g/L xylose, 100 mM Tes-NaOH, 10 mM $MgCl_2$, pH 7.0), to an OD of 0.2. The tubes were placed at 37°C and 250 rpm, sampled at random intervals and analysed by HPLC. Strain CD929 produced 1.7 g/L EtOH and accumulated 1.1 g/L xylulose. Strain CD931 produced 15.2 g/L EtOH while accumulating 4.5 g/L of xylulose. The relative performance of these strains indicates that ethanol formation is about nine times greater when the xylose isomerase gene does not contain the poly-his tag. Similarly, xylulose formation is increased four-fold when the poly-his tail is absent.

Example 12A: Reinsertion of *KmXYL2* gene into strain CD861 (Ex. 8B) by transformation with plasmid pCM52 (Fig. 25); shake flask evaluations of the resulting transformants

The *KmXYL2* gene along with 982 bp of the 5' flank and 200 bp of the 3' flank was amplified from genomic DNA of a wild type *K. marxianus* strain, using primers identified as SEQ. ID. NO. 116 and SEQ. ID. NO. 117. These primers introduce *SacII* restriction sites. The resulting PCR product was digested with *SacII* and ligated to a similarly digested, shrimp alkaline phosphate-treated plasmid pVR113 (Ex. 10A, Fig. 22). The resulting plasmid was designated pCM52 (Fig. 25). Primers identified as SEQ. ID. NO. 118 and SEQ. ID. NO. 119 were used to screen pCM52. The amplified *KmXYL2* gene was sequenced and found to contain two errors, one of which was silent and one causing an amino acid change 85V→I.

Plasmid pCM52 was digested with *PvuII* and transformed into strain CD861 (Ex. 8B) using standard electroporation methods. Transformants were plated onto Sc-Ura plates and incubated at 37°C. Primers identified as SEQ. ID. NO. 104 and SEQ. ID. NO. 105 were used to amplify the coding region of the *KmXYL2* gene and primers identified as SEQ. ID. NO. 120 and SEQ. ID. NO. 121 were used to amplify the region containing the *ScUra3* gene through the 5' flank of and into the coding region of the *KmXYL2* gene. Five transformants positive for both bands and showing *KmXDH* activity (XDH+ transformants) were taken forward for shake flask analysis.

The *KmXDH* activity of the XDH+ transformants shows that the errors introduced into the gene did not destroy its activity.

Isolates from each of the five XDH+ transformants were obtained by passaging them through YPX plates twice. The isolates were then inoculated into YPX medium (pH 6.5) and incubated overnight at 35°C. 2 gcdw of each was harvested by centrifugation and re-suspended in 50 ml 10 g/L yeast extract, 20 g/L peptone, 50 g/L xylose, 10 mM $MgCl_2$ and 7.5 g/L $CaCO_3$. The flasks are put into production at 35°C and 70 rpm shaking. Strain CD861 (Ex. 8B) is similarly cultivated, to compare the effect of the *KmXYL2* re-insertion.

Strain CD861 has a volumetric productivity of 0.46 g/L-hr and a xylose consumption rate of 1.47 g/L-hr. The five XDH+ transformants averaged 70% lower productivity and 65% lower xylose consumption. After ~48 hours, strain CD861 produced ~14 g/L ethanol and ~4.7 g/L xylitol, whereas the five XDH+ transformants produced ~2.8-6.3 g/L ethanol and 1.2-2.2 g/L xylitol. All but one of the XDH+ transformants stopped producing ethanol after about 40 hours, although they all continued to consume xylose linearly past 40 hours.

Cells taken at the start of production and after 67 hours of production are lysed and protein quantification was performed using the advanced protein assay reagent (Cytoskeleton-USA). A 10X solution was prepared by adding 2.355 ml 1M sodium phosphate buffer (pH 7.0) to 5 mg ß-NADH (Sigma), ending in a final NADH concentration of 1.5 mM. Water is added to a volume of 950 µL (less sample volume). Cell free extract is then added and absorbance followed at 340 nm for several minutes to determine background. 50 µL of 0.4 M xylulose was then added and absorbance at 340 nm was followed to determine XDH activity. XDH activity for the five XDH+ transformants taken at the start of production ranged from 287-374 mU/mg. After 67 hours production, XDH activities ranged from 62-86 mU/mg. XDH activity for strain CD861 was 16 mU/mg at the start of production and 3 mU/mg after 67 hours of production.

Example 12B: Deletion of *ScXKS1* gene from strain CD861 (Ex. 8B) by transformation with plasmid pCM28 (Ex. 3B, Fig. 12); shake flask evaluations of the resulting transformants

Plasmid pCM28 was separately linearized with *NaeI* and *PvuII* and transformed into strain CD861 using standard electroporation methods. Cells were recovered in YPD for 3 hours followed by plating onto Sc-Ura dropout media. 32 colonies were re-streaked onto duplicate plates. These were PCR screened using

primers identified as SEQ. ID. NO. 122 and SEQ. ID. NO. 123 to amplify the coding region of the *ScXKS1* gene. Six colonies that failed to produce the band (so indicating the absence of the *ScXKS1* gene) were inoculated overnight in YPD to allow loopout of the *ScUra3* gene, and plated onto FOA plate to select for those in which the loopout event occurred. Colonies that arose were re-streaked onto YPD to select for single colony isolates. One isolate from each transformation was screened using the above primers, and also for the presence of *PXYLA*, *KmXYL1* and *KmXYL2* genes. An additional PCR screening was performed to screen for a double crossover event, in which the *ScXKS1* cassette was replaced with the *PXYLA* cassette from plasmid pCM28. All six isolates tested positive for *PXYLA* and negative for *KmXYL1*, *KmXYL2* and *ScXKS1* genes. One of these was designated CD1065 and sent forward for shake flask evaluation.

Strains CD1065 and CD861 were separately inoculated into 250-ml shake flasks containing 50 ml YPD, and incubated overnight at 37°C and 250 rpm. Cells were harvested and 4 gcdw were inoculated into 50 ml YPX. The flask was incubated at 35°C and shaken at 70 rpm. Samples were withdrawn periodically to monitor fermentation activity. Strain CD1065 exhibits a 10-20 hour time lag during which it consumes xylose very slowly. This is attributed to glucose repression of native xylulokinase genes. After this induction period, the xylose consumption rate increases, and strain CD1065 produces about 13 g/L ethanol after 65 hours. Strain CD861 produces ethanol much more rapidly, producing about 18 g/L ethanol after about 20 hours. Strain CD1065 produces about 3.7 g/L xylulose after about 20 hours, but xylulose concentration decreases gradually thereafter. Xylulokinase activity is about 0 for strain CD1065 and about 417 mU/mg for strain CD861. Xylose consumption rates for strain CD861 are approximately double those of strain CD1065. Xylose isomerase activity is about 0.96 U/mg for strain CD1064 and about 1.79 U/mg for strain CD861. These results indicate that the overexpression of xylulokinase significantly improves xylose utilization and ethanol production rates under these conditions.

Cells from strains CD861 and CD1065 were removed following the production phase, streaked onto YPX plates and placed in an anaerobe jar. Both grew comparably.

Example 12C: Reinsertion of *KmXYL1* gene into strain CD861 (Ex. 8B) by transformation with plasmid pCM55 (Fig. 26); shake flask evaluations of the resulting transformants

The *KmXYL1* gene along with 890 bp of the 5' flank and 414 bp of the 3' flank was amplified from genomic DNA of a wild type *K. marxianus* strain, using primers identified as SEQ. ID. NO. 124 and SEQ. ID. NO. 125. These primers introduce *SacII* restriction sites. The resulting PCR product was digested with *SacII* and ligated to a similarly digested, shrimp alkaline phosphate-treated plasmid pVR113 (Ex. 10A, Fig. 22). The resulting plasmid was designated pCM55 (Fig. 26). Primers identified as SEQ. ID. NO. 118 and SEQ. ID. NO. 126 were used to screen plasmid pCM55, and restriction mapping with *BstBI* and *SbfI* was used to confirm the orientation. The amplified *KmXYL1* gene was sequenced and found to contain three errors, one of which was silent and the others causing amino acid changes 71V→A, 112Y→H and 302I→V.

Plasmid pCM55 was digested with *PvuII* and transformed into strain CD861 (Ex. 8B) using standard electroporation methods. Transformants were plated onto Sc-Ura plates and incubated at 37°C. Primers identified as SEQ. ID. NO. 127 and SEQ. ID. NO. 128 were used to amplify the coding region of the *KmXYL*1 gene and primers identified as SEQ. ID. NO. 121 and SEQ. ID. NO. 129 were used to amplify the region containing the *ScUra3* gene through the 3' flank of and into the coding region of the *KmXYL1* gene. Six transformants positive for both bands (XR+ transformants) were taken forward for shake flask analysis.

Isolates from each of the five XR+ transformants were obtained by restreaking from minimal media onto YPD. The isolates were then re-streaked onto YPX +300 µg/mL G418 + 300 µg/mL hygromycin prior to inoculation in YPX media. These transformants were then inoculated into a medium containing 10 g/L yeast extract, 20 g/L peptone, 50 g/L xylose (pH 6.5) and incubated at 35°C for 48 hours. 1 gcdw of each was harvested by centrifugation and re-suspended in 50 ml 10 g/L yeast extract, 20 g/L peptone, 50 g/L xylose, 10mM $MgCl_2$ and 7.5 g/L $CaCO_3$. The flasks are put into production at 35°C and 70 rpm shaking. Strain CD861 (Ex. 8B) is similarly cultivated, using 2 gcdw, to compare the effect of the *KmXYL1* re-insertion.

Strain CD861 has a volumetric productivity of 0.79 g/L-hr and a xylose consumption rate of 2.02 g/L-hr (based on 2 gcdw). Five of the XR+ transformants exhibited a lag of about 20-50 hours, and thereafter exhibited volume productivities ranged from 0.05-0.13 g/L-hr and xylose consumption rates of 0.45-0.67 g/L-hr.

Ethanol yields for these five XR+ transformants were 18-33%, compared to 51% for strain CD861.

Cells taken at the start of production and after 67 hours of production are lysed and protein quantification was performed using the advanced protein assay reagent (Cytoskeleton-USA). A 100 µL dilution of cell extract was added to a 900 µL aliquot of a solution of 100 mM sodium phosphate, 0.5 M D-xylose and 0.2 mM NADPH equilibrated to 37°C. Absorbance was followed to determine *KmXYL1* activity. Five of the XR+ strains have XR activities in the range of ~34-86 mU/mg. The *KmXYL1* activity of these five XR+ transformants shows that the errors introduced into the gene did not destroy its activity. The *KmXYL1* activity of strain CD861 is approximately 4 mU/mg.

The sixth XR+ transformant shows a *KmXYL1* activity of 19.5 mU/mg, much lower than the others. As such, it resembles strain CD861 much more than the others do. This sixth XR+ strain performs similarly to CD861 on the shake flask cultivation (after an initial lag period) and produces about 13.8 g/L of ethanol after about 51.5 hours.

These results show that native XR activity has an adverse affect on the ability of these strains to ferment xylose to ethanol under these conditions.

Example 12D: Reinsertion of *KmXYX1* and *KmXYL2* genes into strain CD861 (Ex. 8B) by transformation with plasmid pCM58 (Fig. 27); shake flask evaluations of the resulting transformants

A plasmid is constructed in the same manner as described for plasmid pCM52 (Ex. 12A, Fig. 25), except that the *KmXYL2* gene flanks are oriented in the opposite direction than in plasmid pCM52. This plasmid is designated pCM53. The *KmXYL2* gene in plasmid pCM53 is sequenced and found to contain four errors, three of which are silent and the other amounts to a mutation of amino acid 260I→V.

The *KmXYL1* gene along with region of the 5' flank and a region of the 3' flank of the gene was amplified from genomic DNA of a wild type *K. marxianus* strain, using primers identified as SEQ. ID. NO. 130 and SEQ. ID. NO. 131. These primers introduce *SacI* and *NotI* restriction sites. The resulting PCR product was digested with *SacI* and *NotI* and ligated to a similarly digested plasmid pCM53. The resulting plasmid was designated pCM58 (Fig. 27). Primers identified as SEQ. ID. NO. 66 and SEQ. ID. NO. 119 were used to screen plasmid pCM58, and restriction mapping with *ScaI* and *XmnI* was used to confirm the orientation. The amplified *KmXYL1* gene was

sequenced and found to contain two errors, one of which was silent and the other causing an amino acid change 71V→A.

Plasmid pCM58 was digested with *PvuII* and transformed into strain CD861 (Ex. 8B) using standard electroporation methods. Transformants were plated onto Sc-Ura plates and incubated at 37°C. Primers identified as SEQ. ID. NO. 132 and SEQ. ID. NO. 133 were used to amplify the coding region of the *KmXYL*1 gene and primers identified as SEQ. ID. NO. 134 and SEQ. ID. NO. 105 were used to amplify the region containing the *KmXYL2* gene and flanks. Five transformants positive for both bands were taken forward for further analysis.

The five selected transformants were inoculated into a liquid medium containing 10 g/L yeast extract, 20 g/L peptone, 50 g/L xylose (pH 6.5) and incubated at 35°C for 48 hours. 4 gcdw of each was harvested by centrifugation and re-suspended in 50 ml 10 g/L yeast extract, 20 g/L peptone, 50 g/L xylose, 10 mM $MgCl_2$ and 7.5 g/L $CaCO_3$. The flasks are put into production at 35°C and 70 rpm shaking. Strain CD861 (Ex. 8B) is similarly cultivated to compare the effect of the *KmXYL1* and *KmXYL2* re-insertions.

Strain CD861 consumes essentially all the xylose in 30 hours, producing 16.1 g/L ethanol in that time. Ethanol yield for strain CD861 was 67%. The five XR+, XDH+ transformants all consumed xylose much more slowly, and produced average ethanol yields of about 16%. Strain CD861 produced a xylitol yield of 10%, but the five transformants averaged a xylitol yield of 56%. Xylose reductase activity for strain CD861 was about 2 mU/mg, whereas it ranged from 126-425 mU/mg in the transformants, after 73.5 hours cultivation. Xylitol dehydrogenase activity in strain CD861 was zero, but ranged from 21 to 98 mU/mg in the transformants after 73.5 hours cultivation. The increase in activities of these enzymes indicates that the reinserted *KmXYL1* and *KmXYL2* genes were functional in the five transformants. Xylose isomerase activity was higher in strain CD861 (~131 mU/mg) than in the five transformants (~26-45 mU/mg). However, xylitol that was present may have inhibited xylose isomerase activity in the five XR+, XDH+ transformants, resulting in an artificially low value.

This data further indicates that the deletion or disruption of the aldose reductase/xylitol dehydrogenase pathway is beneficial in the strains having an exogenous xylose isomerase gene, under these fermentation conditions.

Example 13A: Construction of XDH-targeting plasmid pMI410 (Fig. 29) for *Candida sonorensis* transformation

Genomic DNA of *C. sonorensis* (ATCC Assession No. 32109) was obtained as described in WO 03/049525.

A portion of the *C. sonorensis* lambda library described in WO 03/049525 was screened by using the *Pichia stipitis* XYL2 gene (Kötter et al. 1990 Curr. Genet, 18: 493-500) as a probe. The XYL2 gene was labeled with 32P -dCTP using the Random Primed Labeling Kit (Boehringer Mannheim). Hybridization was performed by incubation overnight at 55°C in a solution containing 6xSSC 5x Denhardt's 0.5% SDS 100 g/ml denatured herring sperm DNA. The filters were washed after hybridization at room temperature in a solution of 2x SSC for 5 min and repeated, followed a wash at 55C in 2xSSC- 0.1 SDS for 30 minutes. This resulted in isolation of hybridizing clones that contained the *C. sonorensis* xylitol dehydrogenase gene (*CsXDH*).

The 5' region of the *CsXDH* gene was PCR amplified using primers identified as SEQ. ID. NO. 135 and SEQ. ID. NO. 136 and the *CsXDH* gene was used as a template. The PCR product was cut with *SacI* and *SalI* to produce a 642 bp fragment that was gel isolated.

Plasmid pMI281 was prepared by cutting plasmid pMI278 (described in Fig. 14 of WO 03/049525) with *XbaI*, and circularizing a 4976 bp fragment so obtained. The 642 bp fragment form above was ligated to a 4965 bp fragment obtained by digesting plasmid pMI281 with *SacI* and *SalI*. The resulting plasmid was named pMI409 (Fig. 28).

The 3' region of the *CsXDH* gene was PCR amplified using the primers identified as SEQ. ID. NO. 137 and SEQ. ID. NO. 138 and the same lambda library clone as a template. The PCR product was cut with *NotI* and *ApaI*. A 457 bp fragment was gel isolated and ligated to a 5551 bp fragment obtained by digesting plasmid pMI409 with *NotI* and *ApaI*. The resulting plasmid was named pMI410 (Fig. 29).

Example 13B: Construction of XR-targeting plasmid pMI412 (Fig. 31) for *C. sonorensis* transformation

A xylose reductase sequence homologue was amplified by PCR using oligonucleotides identified as SEQ. ID. NO. 139 and SEQ. ID. NO. 140 and genomic DNA of *C. sonorensis* as a template. The oligonucleotides were designed based on conserved sequences found in known fungal xylose reductase and aldose reductase sequences. The 700 bp PCR product was labeled and used as a probe for the isolation

of the genomic *CsXR* lambda clones from the genomic library similarly as described in WO 03/049525.

The 5' region of the *C. sonorensis* xylose reductase (*CsXR*) gene was PCR amplified using primers identified as SEQ. ID. NO. 141 and SEQ. ID. NO. 142. A portion of the *C. sonorensis* lambda library clone described in WO 03/049525 that contains the *CsXR* gene was used as a template. The PCR product was cut with *SacI* and *SalI*. A 526 bp fragment was gel isolated and ligated to a 4965 bp fragment obtained by digesting plasmid pMI281 with *SacI* and *SalI*. The resulting plasmid was named pMI411 (Fig. 30).

The 3' region of the *CsXR* gene was PCR amplified using the primers identified as SEQ. ID. NO. 143 and SEQ. ID. NO. 144 and the same lambda library clone as a template. The PCR product was cut with *NotI* and *ApaI*. A 591 bp fragment was gel isolated and ligated to a 5435 bp fragment obtained by digesting plasmid pMI411 with *NotI* and *ApaI*. The resulting plasmid was named pMI412 (Fig. 31).

Example 13C: Construction of *PXYLA* expression plasmid pMI417 (Fig. 33) for simultaneous insertion of *PXYLA* and deletion of *CsXR* in *C. sonorensis*

The *PXYLA* gene from ATG to the single *AgeI* site was PCR amplified using primers identified as SEQ. ID. NO. 145 and SEQ. ID. NO. 146 and pCM29 (Ex. 8A, Fig. 21) as the template. The PCR product was cut with *Age*I and *Kpn*I and a 453 bp fragment was gel isolated. Plasmid pCM29 was cut with *Age*I. An 8151 bp fragment was gel isolated and partially digested with *Kpn*I. The resulting 6501 bp fragment was gel isolated and ligated to the 453 bp PCR fragment. The plasmid was named pMI400.

Plasmid pMI278 was cut with *BamHI*, filled in with the Klenow enzyme and partially digested with *Xba*I. The resulting 6675 bp fragment was gel isolated. Plasmid pMI400 was cut with *Sbf*I, made blunt ended with T4 polymerase, and cut with *Xba*I. The resulting 1358 bp fragment was gel isolated and ligated to the 6675 bp fragment of pMI278 to form plasmid pMI403 (Fig. 32).

Plasmid pMI412 (Ex. 13B, Fig. 31) was cut with *Sal*I and *Not*I. A 4037 bp fragment was isolated and ligated to a 5042 bp fragment obtained by digesting pM1403 with *Sal*I and *Not*I. The resulting plasmid was named pMI417 (Fig. 33). Plasmid pMI417 contains the *PXYLA* gene and a G418 marker gene with associated promoter and terminator regions between upstream and downstream regions of the

CsXR gene. The *PXYLA* gene is under the control of the *C. sonorensis PGK* promoter and the *ScGAL10* terminator.

Example 13D: Construction of *ScXKS1* expression plasmid pMI425 (Fig. 34) for simultaneous insertion of *ScXKS1* and deletion of *CsXDH* in *C. sonorensis* transformation

The *ScXKS1* 5' region from ATG to the single *BglII* site was PCR amplified using primers identified as SEQ. ID. NO. 147 and SEQ. ID. NO. 148 and pVR103 (Ex. 5C, Fig. 18) as the template. The PCR product was cut with *Not*I and *Bgl*II and a 267 bp fragment was gel isolated. Plasmid pVR103 (Ex. 5C, Fig. 18) was cut with *Not*I and *Bgl*II. A 4633 bp fragment was obtained and ligated to the 267 bp PCR fragment. The plasmid was named pMI406.

Plasmid pMI403 (Ex. 13C, Fig. 32) is cut with *EcoNI*, filled in using the Klenow enzyme and cut with *SalI*. A 6505 bp fragment is gel isolated. Plasmid pMI271 (described in Fig. 7 of WO 03/049525) is cut with *BamHI*, filled in using the Klenow enzyme and cut with *SalI*. The resulting 1666 bp fragment is gel isolated and ligated to the 6505 bp fragment. The resulting plasmid is named pMI423.

Plasmid pMI410 (Ex. 13A, Fig. 29) and plasmid pMI406 were each digested with *XbaI*, dephosphorylated using calf intestinal alkaline phosphatase, and cut with *BamHI*. 5032 bp and 1814 bp fragments were gel isolated. Plasmid pMI423 was digested with *XbaI* and the resulting 2817 bp fragment gel isolated. The three fragments were ligated and the resulting plasmid named pMI425 (Fig. 34). Plasmid pMI425 contains a portion of the promoter region for the *CsXDH* gene, an hph gene with associated promoter and terminator regions, the *ScXKS1* gene under the control of a *C. sonorensis* PGK promoter and a *ScGAL10* terminator, followed by a portion of the terminator region for the *CsXDH* gene.

Example 13E: Generation of *C. sonorensis* mutant strains (Cs/T-1, Cs/T-25, SC/T-34 and Cs/T-51) containing the reconstructed *PXYLA* gene and *ScXKS1* gene

Plasmids pMI417 (Ex. 13C, Fig. 33) and pMI425 (Ex. 13D, Fig. 34) are used to transform a *C. sonorensis* colony using chemical methods similar to those described in WO 03/049525. The transformed cells were plated onto YPD + 200 µg/ml G418 or onto YPD + 200 µg/ml hygromycin and 200 µg/ml G418. PCR analysis with *PXYLA* and *ScXKS1* probes confirms the presence of strains as follows:

Strain Cs/T-1: 1 copy of *PXYLA* and 1 copy of *ScXKS1*

Strain CS/T-25: 1 copy of *PXYLA* and 2 copies of *ScXKS1*

Strain Cs/T-51: 2 copies of *PXYLA* and 1 copy of *ScXKS1*

Strain CS/T-34: 2 copies of *PXYLA* and 2 copies of *ScXKS1*

Example 13F: Generation of *C. sonorensis* mutant strains (Cs/417-201, -206, -208 and -214) containing the reconstructed *PXYLA* gene with and without a deletion of the *CsXR* gene by transformation with plasmid pMI417 (Ex. 13C, Fig. 33)

Plasmid pMI417 is digested with *SacI* and *ApaI* and used to transform a *C. sonorensis* colony using chemical methods similar to those described in WO 03/049525. The transformed cells were plated onto YPD + 200 µg/ml G418. PCR analysis identifies colonies having the *PXYLA* gene and the *CsXR* deletion. Southern analysis confirms that the strain designated Cs/417-206 contains one copy of the *PXYLA* gene whereas the strain designated Cs/417-214 contains two copies of the *PXYLA* gene, in addition to the *CsXR* deletion. Strain Cs/417-201 contains two copies of the *PXYLA* gene but no *CsXR* deletion. Cs/417-208 contains a single copy of the *PXYLA* gene and no *CsXR* deletion.

Example 13G: Generation of a *C. sonorensis* mutant strains (Cs/417-214/425A and Cs/417-214/425B) containing the reconstructed *PXYLA* gene, the *ScXKS1* gene, a deletion of the *CsXR* and with (A) and without (B) deletion of the *CsXDH* gene, by transforming Cs/417-214 (Ex. 13F) with plasmid pMI425 (Ex. 13D, Fig. 34)

Plasmid pMI425 is digested with *PmeI* and *PspOMI* and used to transform strain Cs/417-214 using chemical methods similar to those described in WO 03/049525. The transformed cells were plated onto YPD + 200 µg/ml hygromycin or onto YPD + 200 µg/ml hygromycin + 200 µg/ml G418. PCR and Southern analysis is used to identify colonies that contain the *ScXKS1* gene and to determine whether a deletion of the *CsXDH* gene has occurred. A strain having two copies of the reconstructed *PXYLA* gene, a copy of the *ScXKS1* gene, a deletion of the *CsXR* gene and a deletion of the *CsXDH* gene is designated Cs/417-214/425A. A strain having two copies of the reconstructed *PXYLA* gene, a copy of the *ScXKS1* gene, a deletion of the *CsXR* gene but no deletion of the *CsXDH* gene is designated Cs/417-214/425B.

Example 13H: Generation of *C. sonorensis* mutant strain (C29/403-7) containing the reconstructed *PXYLA* gene and a functional lactate dehydrogenase (LDH) gene by transformation with plasmid pMI403 (Ex. 13C, Fig. 32)

Plasmid pMI403 is used to transform a mutant *C. sonorensis* strain corresponding to that identified as strain 246-27 in WO 03/049525, using chemical

methods similar to those described there. Strain 246-27 contains a functional, exogenous lactate dehydrogenase (LDH) gene. The transformed cells were plated onto YPD + 200 µg/ml G418. A strain exhibiting 70 mU/mg XI activity is designated C29/403-7. It apparently contains multiple copies of the *PXYLA* gene, under the control of the *CsPGK* promoter and *ScGAL10* terminator. In addition, strain C29/403-7 contains the functional lactate dehydrogenase gene.

Example 13I: Generation of *C. sonorensis* mutant strain (C29/403-7/425) containing the reconstructed *PXYLA* gene, the *ScXKS1* gene and a functional LDH gene, by transforming strain C29/403-7 (Ex. 13H) with plasmid pMI425 (Ex. 13D, Fig. 34)

Plasmid pMI425 is digested with *SalI* and *NotI* and used to transform strain C29/403-7 (Example 13H), using chemical methods similar to those described in WO 03/049525. The transformed cells were plated onto YPD + 200 µg/ml hygromycin and onto YPD + 200 µg/ml hygromycin and 200 µg/ml G418. PCR and Southern analysis are used to identify transformants containing the *PXYLA* and *ScSKS1* genes, which are collectively designated C29/403-7/425. Strain C29/403-7/425 contains the functional lactate dehydrogenase gene.

Example 13J: Generation of *C. sonorensis* mutant strains (C29/417-4 and C29/417-9) containing the reconstructed *PXYLA* gene and a deletion of the *CsXR* gene

Plasmid pMI417 (Fig. 13C, Fig. 33) is digested with *SacI* and *ApaI* and used to transform a mutant *C. sonorensis* strain corresponding to that identified as strain 246-27 in WO 03/049525, using chemical methods similar to those described there. The transformed cells were plated onto YPD + 200 µg/ml G418. PCR analysis identifies colonies having the *PXYLA* gene and the *CsXR* deletion. Southern analysis confirms that a mutant strain designated C29/417-4 contains one copy of the *PXYLA* gene, and a mutant strain designated C29/417-9 contains two copies of the *PXYLA* gene, in addition to the *CsXR* deletion.

Example 13K: Generation of *C. sonorensis* mutant strains containing the reconstructed *PXYLA* gene, the *ScXKS1* gene, an LDH gene, a deletion of the *CsXR* gene and with (C29/417-9/425-11) and without (C29/417-9/425-9) deletion the *CsXDH* gene, by transforming strain C29/417-9 (Ex. 13J) with plasmid pMI425 (Ex. 13D, Fig. 34)

Plasmid pMI425 is digested with *PmeI* and *ApaI* and used to transform strain C29/417-9 (Ex. 13J), using chemical methods similar to those described in WO 03/049525. The transformed cells were plated onto YPD + 200 µg/ml hygromycin.

PCR analysis identifies colonies having the *ScXKS1* gene and those also containing the *CsXDH* deletion. Transformants with and without the *CsXDH* deletion are designated C29/417-9/425-11 and C29/417-9/425-9, respectively.

Example 14: Shake flask characterisation of *C. sonorensis* strains from Examples 13E-13K

The following table summarizes strains selected for shake flask characterizations, and reports xylose isomerase and xylulokinase activities.

Ethanol-Producing Strains

Strain Designation	Ex. #	XI[1]	XK[2]	XR[3]	XDH[4]	XI activity (mU/mg)	XK activity (mU/mg)
Cs/T-1	13E	1	1	+	+	170	840
Cs/T-25	13E	1	2	+	+	40	1060
Cs/T-34	13E	2	2	+	+	50	1600
Cs/T-51	13E	2	1	+	+	80	730
Cs/417-214	13F	2	0	–	+	70	70
Cs/417-206	13F	1	0	–	+	30	140
Cs/417-201	13F	2	0	+	+	60	100
Cs/417-208	13F	1	0	+	+	60	120
Cs/417-214/425A	13G	2	1	–	–	50	1440
Cs/417-214/425B	13G	2	1	–	+	50	860

Lactic Acid-Producing Strains (all containing exogenous LDH)

Strain Designation	Ex. #	XI[1]	XK[2]	XR[3]	XDH[4]	XI activity (mU/mg)	XK activity (mU/mg)
C29/403-7	13H	2	0	+	+	90	N.D.[5]
C29/403-7/425	13I	2	1	+	+	90	N.D.
C29/417-4	13J	1	0	–	+	20	250
C29/417-9	13J	2	0	–	+	30	150
C29/417-9/425-11	13K	2	1	–	–	50	960
C29/417-9/425-9	13K	2	1	–	+	30	920

Notes to preceding tables: [1]XI=*PXYLA* gene. [2]XK=*ScXKS1* gene. [3]XR=native xylose reductase gene. [4]XDH=native xylitol dehydrogenase gene. Figures indicate number of integrated copies of the gene. Figures indicate number of integrated copies. "+" indicates the native gene is intact; "–"indicates a deletion of the native gene. [5]N.D.-not determined.

Xylulokinase and xylose isomerase activities for these samples were determined as follows:

The samples (5-10 ml) were centrifuged and washed once with 100 mM sodium phosphate buffer, pH 7.0. After washing, the samples were resuspended into Y-PER yeast protein extraction reagent (Pierce, Rockford, IL) containing protease inhibitors (Complete Mini, EDTA free, Roche). After 20 minutes incubation at room temperature, the cells were centrifuged 13,000 rpm for 10 minutes at +4°C. Supernatant samples were collected for activity measurements.

Xylulokinase activity was determined by a two-step protocol. In step 1, the reaction contained 50 mM HEPES/KOH pH 7.5, 5 mM ATP, 6 mM $MgCl_2$ and 20 mM xylulose. The background reaction was determined by adding water instead of xylose. After adding the enzyme sample, the reactions were incubated at 30°C for 0 and 240 seconds. After the incubation the reactions were stopped by incubating them at 95°C for 5 min. After reaction was stopped 40 mM HEPES/KOH pH 7.5, 10 mM $MgCl_2$, 2.5 mM PEP and 0.2 mM NADH was added to the reaction and absorbance at 340 nm was measured. After measuring the initial absorbance, a mixture of myokinase, pyruvate kinase and lactate dehydrogenase was added. This reaction was incubated further for 1 hour and absorbance at 340 nm was measured. The xylulokinase activity was calculated from the ADP production during the assays.

Xylose isomerase activity was determined by monitoring the oxidation of NADH at 340 nm at 30°C. The reaction contains (in addition to the sample) 100 mM TES-NaOH, pH 7.0, 250 mM xylose, 10 mM $MgCl_2$, 0.6 mM NADH and 2.5 U sorbitol dehydrogenase. The background was detected by measuring the activity without xylose. The xylose isomerase assay was performed in a Cobas Mira automated analyzer (Roche).

Protein concentrations were determined with the Lowry method (Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951), Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193:265-275). Bovine serum albumin (Sigma) was used as a protein standard.

Example 14A: Microaerobic shake flask characterizations of wild-type *C. sonorensis* and strains Cs/T-1, -25, -34, -51 (Ex. 13E), Cs/417-201, -206, -208, -214 (Ex. 13F) and Cs/417-214/425A and -B (Ex. 13G)

50 ml of YP (10 g/L yeast extract and 20 g/L peptone) + 5% glucose + 10mM $MgCl_2$ in 250 ml flasks was inoculated with cells grown on YPD plates and incubated overnight with 250 rpm shaking at 30°C. A 5 ml aliquot was removed for XI, XK and protein assays. OD_{600} was measured and an amount of cells equivalent of OD_{600}=12 (corresponding to 4 g/L cell dry weight) (OD_{600}=40 for strains Cs/417-214/425A and –B) in 50 ml was collected by centrifugation and resuspended in 50 ml of YP + 5% xylose + 10 mM $MgCl_2$. The resuspended cells were transferred into a 250 ml flask containing 1.2 g $CaCO_3$. The cultures were incubated at 30°C with 100 rpm shaking. Samples for HPLC (for measuring xylose consumption and ethanol, xylitol, acetate and xylulose production) and OD_{600} measurements were collected daily. The fermentation was carried out for approximately 11 days.

Strains Cs/T-1, -25, -34 -51 and Cs/417-214/425A and -B produced 2-5 g/L ethanol, with xylitol being the main product for these strains. The wild-type *C. sonorensis* strain and strains Cs/417-201, -206, -208 and -214 did not produce ethanol under the microaerobic conditions. This indicates that both xylose isomerase and xylulokinase are needed for ethanol production under microaerobic conditions. All XR+ strains also produced acetate.

Strains Cs/417-201, -206, -208, -214 and Cs/417-214/425B consumed xylose slowly and no ethanol, xylitol or acetate was produced under these microaerobic conditions. Strain Cs/417-201/425A also consumed xylose slowly and produced some xylitol. At 11 days, strains Cs/417-206 and Cs/417-214 produced 0.7 and 1.2 g/L of xylulose, respectively. This suggests that under these microaerobic conditions,

xylulose accumulated in XI+ strains that did not have overexpressed XK, and that the amount of xylulose accumulation depends on XI activity level.

Example 14B: Anaerobic shake flask characterizations of wild-type *C. sonorensis* and strains Cs/T-25, -34 (Ex. 13E), Cs/417-201, -214 (Ex. 13F), Cs/417-214/425A and B (Ex. 13G)

50 ml of YP + 5% glucose +10 mM $MgCl_2$ in 250 ml flasks was inoculated with cells grown on YPD plates and incubated overnight with 250 rpm shaking at 30°C. A 5 ml aliquot was removed for XI, XK and protein assays. OD_{600} was measured and an amount of cells equivalent of OD_{600}=12 (corresponding to 4 g/L cell dry weight) (OD_{600}=40 for strains Cs/417-214/425A and –B) in 50 ml was collected by centrifugation and resuspended in 50 ml of YP + 5% xylose + 10 mM $MgCl_2$ + 24 g/L $CaCO_3$. The cultures were incubated at 30°C with 100 rpm shaking in 100 ml shake flasks sealed with water locks. The cultivation was sampled. Samples for HPLC (for measuring xylose consumption and ethanol, xylitol, acetate and xylulose production) and OD_{600} measurements were collected periodically. The cultivation was continued for 15 days.

Strains Cs/T-25 and -34 produced 2 g/L ethanol during the first 8 days of incubation whereas the wild-type *C. sonorensis* strain did not produce detectable ethanol. Strains Cs/417-201 and -214 also failed to produce ethanol under these conditions, indicating that both xylose isomerase and xylulokinase genes are needed to obtain anaerobic fermentation ability on xylose in these strains.

Strain Cs/417-214/425A produced ~13 g/L ethanol after 4 days and ~25 g/L ethanol after 11 days. Yield on xylose to ethanol was approximately 55% after 4 days and 53% after 11 days. Xylose consumption was 22-23 g/L after 4 days and 48-49 g/L after 11 days. Strain Cs/417-214/425B consumed ~16 g/L of xylose in 4 days and produced 7 g/L ethanol. This indicates that in these strains, disruption of the native XR/XDH pathway combined with exogenous XI gene expression and XK overexpression is important to achieve good ethanol production. Disruption of both the *CsXR* and *CsXDH* genes is seen to provide the best ethanol production under anaerobic conditions.

Example 14C: Microaerobic shake flask characterizations of LDH-producing *C. sonorensis* mutant strain 246-27 and strains C29/403-7 (Ex. 13H) and C29/403-7/425 (Ex. 13I)

Microaerobic shake flask cultivations were performed for each of these three strains, using the general conditions described in Example 14A, except that lactate production was also monitored.

Under these microaerobic conditions, strain 246-27 and strain C29/403-7 consumed xylose at about 0.5 g/L/hr and all produced lactic acid at about 0.4 g/L/hr. Strain C29/403-7/425 produced about 10-15% more lactic acid and about 10-15% less xylitol than strain C29/403-7 under these conditions. Strain C29/403-7 produced more xylulose than the others, suggesting that xylulose accumulates in this strain because of xylose isomerase activity.

At the end of the cultivations, cells from each flask are streaked onto YP + xylose and onto YP + glucose plates and incubated in anaerobe jars for 9 days. None grew anaerobically, but all grew aerobically in xylose and glucose media.

Example 14D: Anaerobic shake flask characterizations of LDH-producing *C. sonorensis* mutant strain 246-27 and strains C29/403-7 (Ex. 13H), C29/403-7/425 (Ex. 13I) and C29/417-9 (Ex. 13J)

Anaerobic shake flask cultivations were performed for each of these three strains, using the general conditions described in Example 14B, except that lactate production was also monitored.

All consumed xylose at a rate of about 0.1 g/L/hr. Strains 246-27, C29/403-7, C29/403-7/425 and C29/417-9 produced 4.1, 4.8, 6.4 and 3.0 g/L of lactic acid, 0.3, 0.45, 0.3 and 0.3 g/L xylitol and 0.3, 1.45, 0.9 and 0.85 g/L xylulose, respectively, after 141 hours. Under these conditions, the overexpression of the xylulokinase enzyme leads to improved lactic acid production. Strains with overexpressed xylose isomerase but without overexpressed xylulokinase accumulate xylulose, indicating that the xylose isomerase gene is active.

Example 14E: Microaerobic shake flask characterizations of LDH-producing *C. sonorensis* mutant strain 246-27 and strains C29/417-4 and -9 (Ex. 13J), C29/417-9/425-9 and -11 (Ex. 13K)

Microaerobic shake flask cultivations were performed for each of these three strains, using the general conditions described in Example 14C. Cultivations were continued for 7 days.

Under these microaerobic conditions, strain 246-27 consumed xylose faster than the other strains. Strain C29/417-4, -9, C20/417-425-9 and -11 produced about

2-5 g/L lactic acid after 7 days, after which time 25-35 g/L residual xylose remained. The ability of strain C29/417-9/425-11 to produce lactic acid confirms that the xylose isomerase and xylulokinase pathway is operative in these strains. Strain C29/417-9/425-11 consumed xylose slightly faster than the C29/417-9/425-9 strain, but also accumulated 1-2 g/L xylitol.

Example 14F: Anaerobic shake flask characterizations of LDH-producing *C. sonorensis* mutant strains C29/417-9 (Ex. 13J), C29/417-9/425-9 and -11 (Ex. 13K)

Anaerobic shake flask cultivations were performed for each of these three strains, using the general conditions described in Example 14D.

Strains C29/417-9, C29/417-9/425-9 and C29/417-9/425-11 produced 4.4, 6.2 and 24.4 g/L lactic acid after 146 hours. Yield of lactic acid on xylose was 0.40, 0.70 and 0.95 g/g, respectively for these strains. No xylitol was produced by either strain C29/417-9/425- 9 or -11, whereas strain C29/417-9 produced 2.7 g/L xylitol. Under these conditions, the overexpression of the xylulokinase enzyme leads to improved lactic acid production. This indicates that in these strains, disruption of the native xylose reductase/xylitol dehydrogenase pathway combined with exogenous xylose isomerase and xylulokinase overexpression provides good lactic acid production. Disruption of both the *CsXR* and *CsXDH* genes is seen to provide the best production under anaerobic conditions.

Example 15A: Generation of *K. marxianus* mutant strains (CD1103 and CD1106) containing the *Cyllamyces aberensis* xylose isomerase (*CaXYLA*) gene

Cyllamyces aberensis DNA (isolate ffew1 from cow, U.K.) was obtained from Gareth Wyn Griffith, Institute of Biological Sciences, University of Wales, Aberystwyth, Ceredigion SY23 3DA, Great Britain. PCR reactions were conducted using this DNA as the template, using 0.5 µM of the sense primer identified as SEQ. ID. NO. 149 and 0.5 µM of the antisense primer identified as SEQ. ID. NO. 150 Phusion HF buffer and 0.2 mM of each dNTP. In this construct, the first methionine encoded as detected at the 5' end sequence of the gene served as the initiation methionine, and an in-frame stop codon was included in addition to *SbfI* restriction sites. Before 3 minutes denaturation, 2 U of Phusion polymerase (Finnzymes Oy, Espoo, Finland) was added. The reaction was cycles 35 times as follows: 10 seconds at 98°C, 30 seconds at 45°C and 1 minute at 72°C with a final extension of 8 minutes at 72°C. A 1346 bp PCR fragment was obtained and ligated to TOPO plasmid with

the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and sequenced. The *C. aberensis* xylose isomerase (*CaXYLA*) gene has the nucleotide sequence identified as SEQ. ID. NO. 151. The deduced amino acid sequence for the protein encoded by this gene is given as SEQ. ID. NO. 152.

K. marxianus genomic DNA was obtained from a wild-type *K. marxianus* strain similar to that described before. The *K. marxianus* Ura3 gene *(KmURA3)* gene plus about 750 bp of the Ura3 promoter region and about 250 bp to the Ura3 terminator region was isolated in a standard protocol using Failsafe Polymerase (Epicenter), with the genomic DNA as template and primers identified as SEQ. ID. NO. 153 and SEQ. ID. NO. 154. PCR conditions were 5 minutes at 95°C, 15 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 2 minutes at 68°C, followed by 25 cycles of 30 seconds at 95°C and 2.5 minutes at 68°C, ending in a blunt end generation cycle of 68°C for 8 minutes. The resulting ~1.8 kb PCR product was cloned into pCR-XL-TOPO vector (Invitrogen) and sequenced for verification. The resulting plasmid was designated pSO90. The nucleotide sequence of the cloned *KmURA3* gene appears as SEQ. ID. NO. 155. Plasmid pSO90 is digested with *SphI* and the ~1.8 kbp *KmURA3* region is gel isolated and ligated into a similarly digested and shrimp alkaline phosphatase treated ~4570 bp fragment of plasmid pCM9 (Ex.1I, Fig. 9). The resulting plasmid (pSO91, Fig. 35) contains the *KmURA3* selection gene, the *KmPDC1* promoter, a *SbfI* site and the *ScCYC1* terminator.

The *CaXYLA*-containing plasmid from above was digested and a ~1.4 kbp fragment containing the *CaXYLA* gene is gel isolated. Plasmid pSO91 is similarly digested and shrimp alkaline phosphate treated to obtain a 6376 bp fragment. These fragments are ligated with HC T4 Ligase (Invitrogen) to form a plasmid (pSO99, Fig. 36) that contains a *KmUra3* selection gene and the *CaXYLA* gene under control of the *KmPDC1* promoter and *ScCYC1* terminator.

A *K. marxianus* colony corresponding to CD 806 (Ex. 6B) is cultured. 20 ml of cells are spun down and transformed with plasmid pSO99 using standard electroporation methods, after digestion of the plasmid with *AatII* and *BsmBI* (without clean-up). 100µL of cells were plated on SC-Ura plates and allowed to grow at 37°C until colonies formed. The colonies were streaked onto secondary Sc-Ura plates, where all exhibited secondary growth. The transformants were screened by PCR for the presence of the intact *KmURA3* gene (inserted with plasmid pSO99) with primers identified as SEQ. ID. NO. 156 and SEQ. ID. NO. 157 and also for an

internal region of the *CaXYLA* gene using primers identified as SEQ. ID. NO. 158 and SEQ. ID. NO. 159. CD1103 contains the *CaXYLA* gene, an intact *KmURA3* gene, and a disrupted *KmURA3* gene. CD1106 contains the *CaXYLA* gene and the disrupted *KmURA3*.

Example 15B: Anaerobic fermentation with strains CD1103 and CD1106 (Ex. 15A)

Baffled shake flasks containing 100 ml of media (20 g/L yeast extract, 10 g/L peptone, 100 g/L glucose) were separately inoculated with strains CD1103 and 1106 were incubated overnight at 35°C and shaking at 250 rpm. Cells were harvested after ~14 hours, and 4 g/L cell dry weight of each strain were added to separate 50 ml Falcon screw top tubes containing 45 ml yeast peptone, 50 g/L D-xylose, 7.5 g/L $CaCO_3$ and 10 mM $MgCl_2$. Tubes were cultivated anaerobically for 65 hours at 30°C with shaking at 70 rpm. Strain 1103 produced over 9 g/L of ethanol, and strain 1106 produced over 7 g/L ethanol in that time. XI activity was determined by lysing cells taken from the growth phase. XI activity was measured and found to be approximately 83 mU/mg for each of strains 1103 and 1106.

Example 16A: Cloning *B. thetaiotaomicron* xylose isomerase (*BtXYLA*) gene (pSO89, Fig. 37)

Bacteroides thetaiotaomicron genomic DNA was obtained from Washington University (St. Louis, MO) Department of Molecular Biology and Pharmacology. The *BtXLYA* gene was isolated in a standard protocol using Pfx Polymerase (Stratagene), with the genomic DNA as template and primers identified as SEQ. ID. NO. 160 and SEQ. ID. NO. 161. PCR conditions were 95°C for 3 minutes; 15 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 2 minutes at 68°C, followed by 20 cycles of 30 seconds at 95°C and 2.5 minutes at 68°C, ending in a blunt end generation cycle of 68°C for 8 minutes. The resulting ~1.4 kb PCR product was cloned into pCR-XL-TOPO vector (Invitrogen) and sequenced for verification. The resulting plasmid was designated pSO89 (Fig. 37). The nucleotide and deduced amino acid sequences of the cloned *BtXYLA* gene appear as SEQ. ID. NO. 162 and SEQ. ID. NO. 163, respectively.

Example 16B: Creation of plasmid containing *BtXYLA* and *KmURA3* selection gene plasmid (pSO96, Fig. 38)

The ~1.4 kb *BtXYLA* gene was gel extracted from plasmid pSO89 after an *SbfI* digest and ligated to a similarly digested 6376bp fragment of plasmid pSO91 (Ex. 16A, Fig. 35). The resulting plasmid (pSO96, Fig. 38) contains the *KmURA3*

selection gene, the *BtXYLA* gene under the control of the *KmPDC1* promoter, and *ScCYC1* terminator.

Example 16C: Creation of *K. marxianus* mutants (CD1089-1096) by transforming a strain corresponding to CD806 (Ex. 6B) with plasmid pSO96 (Ex. 16B, Fig. 38).

A *K. marxianus* colony corresponding to CD 806 (Ex. 6B) is cultured. 20 ml of cells are spun down and transformed with plasmid pSO96 using standard electroporation methods. Plasmid pSO96 is digested with *AatII* and *BsmBI* prior to integration. 100 µL of cells were plated on Sc-Ura plates and allowed to grow at 37°C until colonies formed. The colonies were streaked onto secondary plates of SC-Ura. The transformants were screened by PCR for the presence of the intact *KmURA3* gene with primers identified as SEQ. ID. NO. 156 and SEQ. ID. NO. 157. A PCR screening for a ~450 bp region upstream of the *BtXYLA* stop codon and just inside of the *ScCYC1* terminator is performed with primers identified as SEQ. ID. NO. 164 and SEQ. ID. NO. 165. Of the transformants testing positive for both the native *KmURA3* and *BtXYLA* gene, eight are selected and designated CD1089-1096, respectively.

For determination of xylose isomerase activity, strains were grown at 30°C, 250 rpm for ~14 hours in YPD (10 g/L yeast extract, 20 g/L peptone, 100 g/L dextrose). Cells were lysed according to protocol for Y-PER (Pierce #78990). Total protein was determined using Advanced Protein Assay Reagent (Cysoskeleton #ADV01) with BSA as a standard. Xylose isomerase activities for strains CD 806 and CD1089-1096 are as follows:

Strain	XI activity (mU/mg)
CD806 (Ex. 6B)	0
CD1089	143±13
CD1090	48±1
CD1091	41±6
CD1092	108±21
CD1093	45
CD1094	74±29
CD1095	10±13
CD1096	87±34

The higher activities of CD1089 and CD1092 may be due to the integration of multiple copies of the *BtXYLA* gene or a preferred site of integration.

Example 16D: Shake flask characterisation of strains CD1089-1096 (Ex. 16C).

Single colonies of strains CD806, and CD1089-1096 were separately inoculated for growth in 100ml media consisting of 10 g/L yeast extract, 20 g/L peptone, and 100 g/L dextrose. The cells were grown for 14 hours at 30°C, after which the cells were collected and cell dry weight determined. 1.4 g/L cell dry weight of each culture is added to separate 50 mL Falcon tubes containing 10g/L yeast extract, 20g/L peptone, 50g/L D-xylose, 7.5 g/L $CaCO_3$, and 10mM $MgCl_2$. These cultures were incubated at 30°C with shaking at 70 rpm. Broth samples were taken for HPLC analysis approximately every 24 hours. After 72 hours, results of this anaerobic fermentation were as shown in the following table.

Strain	Xylose Consumed (g)	Ethanol Produced (g)	Xylitol Produced (g)
CD806 (Ex. 6B)	~2.5	~0.4	~0
CD1089	~19.6	~7.3	~1.45
CD1090	~10.7	~4.0	~0.9
CD1091	~11.0	~4.15	~0.9
CD1092	~16.55	~6.2	~1.1
CD1093	~7.5	~4.1	~0.9
CD1094	~9.6	~3.7	~0.9
CD1095	~1.7	0	0
CD1096	~9.45	~3.95	~0.9

In microaerobic shake flask fermentation studies, strains CD1089 and CD1092 produced up to about 1 gram of ethanol after about 7 hours fermenting at 30°C and 70 rpm.

Strains CD1089-1096 were plated at the end of a low oxygen xylose cultivation onto YPX plates and placed in an anaerobic chamber for two days. All except CD1095 exhibited anaerobic growth under these conditions, with strains CD1089 and CD1092 showing the greatest anaerobic growth.

Example 17: Shake-flask fermentation of strains CD804 (Ex. 4D), CD805 (Ex. 5E) and CD806 (Ex. 6B) in media with xylose and externally added commercial enzyme glucose isomerase

Strains CD804, CD805 and CD806 were separately inoculated to an OD_{600} of 0.1 from YPD agar plates and were grown for 16 hours at 33°C with 250 rpm, in 250

mL baffled shake flasks containing 50 mL YPD supplemented with 50 μg/ml G418. After determining that residual glucose remained in each flask and that the cells were consequently in exponential growth phase, 0.5 g/L cell dry weight equivalents of each strain were harvested by centrifugation and separately resuspended in 250 mL baffled shake flasks containing 47.5 mL YP supplemented with 50 g/L D-xylose. 2.5 ml glucose isomerase (Gensweet SGI; Genencor Inc., CA) (also known as xylose isomerase) was added to the shake-flasks and the cultures were grown at 33°C with 70 rpm. For controls, 0.5 g/L cell dry weight equivalents of strains each of strains CD804, 805 and 806 were separately resuspended in 250 mL baffled shake flasks containing 50 mL YP supplemented with 50 g/L D-xylose where the glucose isomerase was omitted. These shake-flasks were also incubated at 33°C with 70 rpm. Samples were withdrawn at various time intervals and the cells were removed by filtration. Culture supernatant was analyzed for xylose, xylitol and ethanol by HPLC methods.

After 25 hours, strain CD804 (Ex. 4D) had consumed 15 g/L D-xylose and produced about 4.7 g/L of xylitol and 1 g/L of ethanol from the flask containing the glucose isomerase. In contrast, strains CD805 (Ex. 5C) and CD806 (Ex. 6B) had each consumed 25 g/L D-xylose in the presence of glucose isomerase. Strain CD805 produced in this time about 1.9 g/L of xylitol and 7.1 g/L of ethanol. Strain CD806 produced in this time about 1.8 g/L of xylitol and 6.8 g/L of ethanol. Xylulose seems to be consumed by the strains at very high rates, something which is not observed in *S. cerevisiae*. The non-oxidative pentose phosphate pathway controls the fermentation rate of xylulose but not of xylose in *S. cerevisiae* TMB3001. FEM Yeast Res. 2002 Aug; 2(3):277-82. Johansson B, Hahn-Hagerdal B). Without added glucose isomerase, each of strains CD804, CD805 and CD806 consumed xylose very slowly.

Example 18: Transformation of strain corresponding to CD806 (Ex. 6B) with self-replicating plasmid pCM48 (Fig. 40) to introduce *PXYLA* gene; cultivation of the resulting strains

A cassette containing the *KmPDC1* promoter, *PXYLA* gene and *ScCYC1* terminator was PCR amplified using primers identified as SEQ. ID. NO. 166 and SEQ. ID. NO. 167, using plasmid pCM29 (Ex. 8A, Fig. 21) as the template. These primers were designed to incorporate *PacI* and *MluI* restriction sites. Thermocycling conditions were an initial incubation of 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 70°C for 3 minutes. This was followed

by a final incubation at 70°C for 8 minutes. The product was digested with the above restriction enzymes and a 2.804 kbp fragment so obtained was ligated into fragment obtained by similarly digesting a plasmid designated pSO57 (Fig. 39) (containing a pKD1 self-replication site), to yield the plasmid pCM48 (Fig. 40). The transformants were restriction mapped using *EcoR*I and *Sbf*I, and two were taken forward for sequencing. The entire *PXYLA* coding region was sequenced and verified to be identical for both transformants to the sequence on plasmid pCM29.

2 μg of undigested plasmid pCM48 was transformed into a strain corresponding to CD806 (Ex. 6B) using to standard electroporation methods. Cells were recovered for four hours in YPX, plated on YPX plates containing 300 μg/ml G418 and 150 μg/ml hygromycin and incubated at 37°C for 2 days. This produced a large number of transformants. Several transformants were re-streaked onto identical plates and incubated at 37°C for several days. Four transformants were selected and inoculated into ~100 ml of YPX in a 250 ml-baffled shake flask and incubated at 37°C with 250 rpm shaking. Strain CD861 was included and biomass prepared in the same manner. After 17 hours, 2 gcdw of each was inoculated into separate 250 mL-baffled shake flasks containing 50 mL media (10 g/L yeast extract, 20 g/L yeast peptone, 50 g/L xylose, 7.5 g/L $CaCO_3$, 10 mM $MgCl_2$, and pH 7.0). The four transformants produced about 9-12.3 g/L ethanol after about 40 hours. The parent strain produced no ethanol.

10 mL of the overnight culture was harvested by centrifugation and taken forward to enzyme assays. Xylose isomerase activities for the four transformants ranged from 456 to 531 mU/mg.

We claim:

1. A genetically modified yeast cell having a genome and a functional, exogenous xylose isomerase gene, wherein the exogenous xylose isomerase gene is operatively linked to promoter and terminator sequences that are functional in the yeast cell, and the modified yeast cell further has a deletion or disruption of a native gene that produces an enzyme that catalyzes the conversion of xylose to xylitol.

2. The genetically modified yeast cell of claim 1 wherein the functional, exogenous xylose isomerase gene is integrated into the genome of the yeast cell.

3. The genetically modified yeast cell of claim 1 or 2, wherein the enzyme that catalyzes the conversion of xylose to xylitol is specific to the conversion of xylose to xylitol.

4. The genetically modified yeast cell of any of claims 1-3, wherein the native gene that produces an enzyme that catalyzes the conversion of xylose to xylitol is a functional xylose reductase gene.

5. The genetically modified yeast cell of any of claims 1-4, further having a deletion or disruption of a functional native xylitol dehydrogenase gene.

6. The genetically modified yeast cell of any of claims 1-5, which overexpresses a functional xylulokinase.

7. The genetically modified yeast cell of claim 6, which contains a functional, exogenous xylulokinase gene operatively linked to promoter and terminator sequences that are functional in the yeast cell.

8. The genetically modified yeast cell of claim 7, wherein the functional, exogenous xylulokinase gene is a *S. cerevisiae* xylulokinase gene.

9. The genetically modified yeast cell of any of claims 1-8 that naturally grows on xylose.

10. The genetically modified yeast cell of claim 9, which has an active native pathway from xylulose-5-phosphate to glyceraldehyde-3-phosphate.

11. The genetically modified yeast cell of any of claims 1-11 that is of the genera *Kluyveromyces, Candida, Pichia, Brettanomyces, Pachysolen* or *Saccharomyces.*

12. The genetically modified yeast cell of any of claims 1-11, wherein the xylose isomerase gene is at least 95% homologous to SEQ. ID. NO. 58 or 151.

13. The genetically modified yeast cell of any of claims 1-11, wherein the xylose isomerase gene encodes a xylose isomerase enzyme that is at least 95% homologous to SEQ ID. NOs. 59 or 152.

14. The genetically modified yeast cell of any of claims 1-11, wherein the xylose isomerase gene encodes a xylose isomerase enzyme that is at least 98% homologous to SEQ. ID. NO. 59.

15. The genetically modified yeast cell of any of claims 1-11, wherein the xylose isomerase gene encodes a xylose isomerase enzyme that is at least 98% homologous to SEQ. ID. NO. 152.

16. The genetically modified yeast cell of any of claims 1-11, wherein the xylose isomerase gene is a bacterial xylose isomerase that is at least 60% homologous to SEQ. ID. NO. 162.

17. The genetically modified yeast cell of any of claims 1-11, wherein the xylose isomerase gene is a bacterial xylose isomerase that encodes a xylose isomerase enzyme that is at least 95% homologous to SEQ. ID. NO. 163.

18. The genetically modified yeast cell of any of claims 1-11, wherein the xylose isomerase gene is as identified in SEQ. ID. NO. 58

19 The genetically modified yeast cell of any of claims 1-11, wherein the xylose isomerase gene is as identified in SEQ. ID. NO. 151.

20. The genetically modified yeast cell of any of claims 1-11, wherein the xylose isomerase gene is as identified in SEQ. ID. NO. 162.

21. The genetically modified yeast cell of any of claims 1-20 wherein the yeast cell is *K. marxianus.*

22. The genetically modified yeast cell of any of claims 1-20 wherein the yeast cell is *C. sonorensis.*

23. A genetically modified yeast cell of the genera *Kluyveromyces* or *Candida,* having integrated into its genome a functional, exogenous xylose isomerase gene, wherein the exogenous xylose isomerase gene is operatively linked to promoter and terminator sequences that are functional in the yeast cell.

24. A genetically modified yeast cell having a genome and a functional, exogenous xylose isomerase gene, wherein the exogenous xylose isomerase gene is operatively linked to promoter and terminator sequences that are functional in the yeast cell, and which further contains a functional, exogenous xylulokinase gene operatively linked to promoter and terminator sequences that are functional in the yeast cell.

25. The genetically modified yeast cell of claim 24, wherein the exogenous xylose isomerase gene and the exogenous xylulokinase gene are integrated into the genome of the yeast cell.

26. A genetically modified yeast cell having a deletion or disruption of a functional, native gene that produces an enzyme that catalyzes the reaction of xylitol to xylulose or of xylulose to xylitol.

27. The genetically modified yeast cell of claim 26, wherein the enzyme that catalyzes the conversion of xylitol to xylulose is specific to the conversion of xylitol to xylulose.

28. The genetically modified yeast cell of claim 26 or 27 which further contains a functional, exogenous xylulokinase gene operatively linked to promoter and terminator sequences that are functional in the yeast cell.

29. The genetically modified yeast cell of any of claims 26-28, wherein the gene that produces an enzyme that catalyzes the reaction of xylitol to xylulose or of xylulose to xylitol is a xylitol dehydrogenase gene, and the yeast cell further has integrated into its genome a functional, exogenous xylose isomerase gene, wherein the exogenous xylose isomerase gene is operatively linked to promoter and terminator sequences that are functional in the yeast cell.

30. The genetically modified yeast cell of any of claims 23, 24 or 29, wherein the xylose isomerase gene is at least 95% homologous to SEQ. ID. NO. 58 or 151.

31. The genetically modified yeast cell of any of claims 23, 24 or 29, wherein the xylose isomerase gene encodes a xylose isomerase enzyme that is at least 95% homologous to SEQ ID. NO. 59 or 152.

32. The genetically modified yeast cell of any of claims 23, 24 or 29, wherein the xylose isomerase gene encodes a xylose isomerase enzyme that is at least 98% homologous to SEQ. ID. NO. 59.

33. The genetically modified yeast cell of any of claims 23, 24 or 29, wherein the xylose isomerase gene encodes a xylose isomerase enzyme that is at least 98% homologous to SEQ. ID. NO. 152.

34. The genetically modified yeast cell of any of claims 23, 24 or 29, wherein the xylose isomerase gene is a bacterial xylose isomerase gene that is at least 60% homologous to SEQ. ID. NO. 162.

35. The genetically modified yeast cell of any of claims 23, 24 or 29, wherein the xylose isomerase gene is a bacterial xylose isomerasge gene that encodes a xylose isomerase enzyme that is at least 60% homologous to SEQ. ID. NO. 163.

36. A genetically modified yeast cell having a deletion or disruption of a native gene that produces an enzyme that catalyzes the conversion of xylose to xylitol.

37. The genetically modified yeast cell of claim 36 wherein the enzyme is specific to the conversion of xylose to xylitol.

38. The genetically modified yeast cell of claim 36 or 37 further having a deletion or disruption of a native gene that produces an enzyme that catalyzes the conversion of xylitol to xylulose or of xylulose to xylitol.

39. The genetically modified yeast cell of any of claims 36-38 further wherein the gene that produces an enzyme that catalyzes the conversion of xylitol to xylulose or of xylulose to xylitol is a xylitol dehydrogenase gene.

40. The genetically modified yeast cell of any of claims 24-39 that is of the genera *Kluyveromyces, Candida, Pichia, Brettanomyces, Pachysolen* or *Saccharomyces.*

41. The genetically modified yeast cell of any of claims 23-40 wherein the yeast cell is *K. marxianus.*

42. The genetically modified yeast cell of any of claims 23-40 wherein the yeast cell is *C. sonorensis.*

43. The genetically modified yeast cell of any of claims 1-42, further having a deletion or disruption of a native pyruvate decarboxylase gene.

44. The genetically modified yeast cell of any of claims 1-43, further having integrated into its genome a functional, exogenous lactate dehydrogenase gene, wherein the exogenous lactate dehydrogenase gene is operatively linked to promoter and terminator sequences that are functional in the yeast cell.

45. The modified yeast cell of any of claims 1-55 which has a capacity to transport xylose through its cell wall or membrane.

46. A fermentation process in which a cell of any of claims 1-45 is cultured under fermentation conditions in a fermentation broth that includes a pentose sugar.

47. A fermentation process in which a cell of any of claims 1-42 is cultured under fermentation conditions in a fermentation broth that includes a pentose sugar, and ethanol is produced as a major fermentation product.

48. A fermentation process in which a cell of claim 44 is cultured under fermentation conditions in a fermentation broth that includes a pentose sugar, and lactate is produced as a major fermentation product.

49. The fermentation process of any of claims 46-48 in which the pentose sugar includes xylose.

50. The fermentation process of any of claims 46-49 in which the fermentation broth further includes a hexose sugar.

51. The fermentation process of any of claims 46-50 which is an anaerobic fermentation.

52. A fermentation process in which the cell of any of claims 26-28 or 36-39 is cultured under fermentation conditions in a fermentation broth that includes a pentose sugar and xylose isomerase.

53. The fermentation process of claim 52 wherein the pentose sugar includes xylose.

54. A vector for transforming a host yeast cell, comprising, in order of transcription, an upstream (5'-) region of a xylose reductase gene of the host cell, a selection marker expression cassette, and a downstream (3'-) region of a xylose reductase gene of the host cell.

55. The vector of claim 54, wherein the marker expression cassette includes a gene that confers resistance to hygromycin, G418 or ampicillin resistance gene, or a Ura3 gene.

56. The vector of claim 55, wherein the marker expression cassette includes a G418 resistance gene under the control of a *ScPDC1* promoter and *ScGAl10* terminator.

57. The vector of claim 55, wherein the marker expression cassette includes a hisG-Ura3-hisG cassette.

58. The vector of any of claims 54-57, further comprising a xylose isomerase expression cassette between the upstream and downstream regions of the xylose reductase gene of the host cell.

59. The vector of claim 58, wherein the xylose isomerase expression cassette includes a promoter that is native to the host yeast cell.

60. The vector of claim 58 or 59, wherein the xylose isomerase expression cassette includes a *ScCYC1* or *ScGAL10* terminator.

61. The vector of any of claims 54-60, further comprising a xylulokinase expression cassette between the upstream and downstream regions of the xylose reductase gene of the host cell.

62. The vector of claim 61, wherein the xylulokinase expression cassette includes a promoter that is native to the host cell, or a *ScTEF1* promoter.

63. The vector of claim 61 or 62, wherein the xylulokinase expression cassette includes a *ScCYC1* or *ScGAL10* terminator.

64. The vector of any of claims 61-63, wherein the xylulokinase gene is a *S. cerevisiae* xylulokinase gene.

65. A vector for transforming a host yeast cell, comprising, in order of transcription, an upstream (5'-) region of a xylitol dehydrogenase gene of the host cell, a selection marker expression cassette, and a downstream (3'-) region of a xylose dehydrogenase gene of the host cell.

66. The vector of claim 65, wherein the marker expression cassette includes a gene that confers resistance to hygromycin, G418 or ampicillin, or a Ura3 gene.

67. The vector of claim 66, wherein the marker expression cassette includes a G418 resistance gene under the control of a *ScPDC1* promoter and *ScGAl10* terminator.

68. The vector of claim 66, wherein the marker expression cassette includes a hisG-Ura3-hisG cassette.

69. The vector of any of claims 65-68, further comprising a xylose isomerase expression cassette between the upstream and downstream regions of the xylitol dehydrogenase gene of the host cell.

70. The vector of claim 69, wherein the xylose isomerase expression cassette includes a promoter that is native to the host yeast cell cassette.

71. The vector of claim 69 or 70, wherein the xylose isomerase expression cassette includes a *ScCYC1* or *ScGAL10* terminator.

72. The vector of any of claims 65-71, further comprising a xylulokinase expression cassette between the upstream and downstream regions of the xylitol dehydrogenase gene of the host cell.

73. The vector of claim 62, wherein the xylulokinase expression cassette includes a promoter that is native to the host cell, or a *ScTEF1* promoter.

74. The vector of claim 72 or 73, wherein the xylulokinase expression cassette includes a *ScCYC1* or *ScGAL10* terminator.

75. The vector of any of claims 72-74, wherein the xylulokinase gene is a *S. cerevisiae* xylulokinase gene.

76. A vector for transforming a host yeast cell, comprising a *HisG-Ura3-HisG* cassette, and a xylose isomerase expression cassette, a xylulokinase expression cassette or both a xylose isomerase expression cassette and a xylulokinase expression cassette.

77. A vector for transforming a yeast cell, comprising a xylose isomerase expression cassette including a *K. marxianus* promoter or a *C. sonorensis* promoter, and a marker expression cassette.

78. The vector of claim 77, wherein the *K. marxianus* or *C. sonorensis* promoter is a PDC or PGK promoter.

79. The vector of claim 77 or 78, wherein the xylose isomerase expression cassette includes a *K. marxianus, C. sonorensis* or *S. cerevisiae* terminator.

80. The vector of claim 79, wherein the terminator is a *ScCYC1* or *ScGAL10* terminator.

81. The vector of any of claims 77-80, wherein the marker expression cassette includes a gene that confers resistance to hygromycin, G418 or ampicillin, or a Ura3 gene.

82. The vector of claim 81, wherein the marker expression cassette includes a G418 resistance gene under the control of a *ScPDC1* promoter and *ScGAl10* terminator.

83. The vector of claim 81, wherein the marker expression cassette includes a *HisG-Ura3-HisG* cassette.

84. The vector of any of claims 76-83, further comprising a xylulokinase expression cassette.

85. The vector of claim 84, wherein the xylulokinase expression cassette includes a promoter that is native to the host cell, or a *ScTEF1* promoter.

86. The vector of claim 84 or 85, wherein the xylulokinase expression cassette includes a *ScCYC1* or *ScGAL10* terminator.

87. The vector of any of claims 84-86, wherein the xylulokinase gene is a *S. cerevisiae* xylulokinase gene.

88. A vector for transforming a host yeast cell comprising a xylulokinase expression cassette and a marker expression cassette.

89. The vector of claim 88, wherein the xylulokinase expression cassette includes a *K. marxianus* promoter, a *C. sonorensis* promoter or a *S. cerevisiae* promoter.

90. The vector of claim 89, wherein the xylulokinase expression cassette includes a *K. marxianus* or *C. sonorensis* PDC or PGK or a *S. cerevisiae* PDC, PGK or TEF1 promoter.

91. The vector of any of claims 88-90, wherein the xylulokinase expression cassette includes a *ScCYC1* or *ScGAL10* terminator.

92. The vector of any of claims 88-91, wherein the marker expression cassette includes a gene that confers resistance to hygromycin, G418 or ampicillin, or a Ura3 gene.

93. The vector of any of claims 88-92, wherein the marker expression cassette includes a hisG-Ura3-hisG cassette.

94. A vector of any of claims 54-93, further including an origin of replication that allows the vector to replicate and be maintained in the host yeast cell.

95. A genetically modified yeast cell having a functional, exogenous xylose isomerase gene integrated into its genome, which cell has a xylose reductase activity of no greater than 10 mU/mg.

96. The genetically modified yeast cell of claim 95, further having a xylitol dehydrogenase activity of no greater than 2 mU/mg.

97. A genetically modified yeast cell having a functional, exogenous xylose isomerase gene integrated into its genome, which cell has a xylitol dehydrogenase activity of no greater than 2 mU/mg.

98. A genetically modified yeast cell having a functional, exogenous xylose isomerase gene integrated into its genome, which cell has a xylulokinase activity of at least 100 mU/mg.

99. The genetically modified yeast cell of claim 98, further having a xylitol dehydrogenase activity of no greater than 2 mU/mg.

100. The genetically modified yeast cell of claim 98 or 99, further having a xylose reductase activity of no greater than 10 mU/mg.

101. A method of evaluating xylose isomerase genes for activity in a host cell, comprising transforming a host cell having low aldose reductase enzyme activity, low xylitol dehydrogenase enzyme activity and overexpressed xylulokinase enzyme activity, with a vector containing the xylose isomerase gene under the control of promoter and terminator which are active in the host cell, selecting for transformed cells containing the xylose isomerase gene, and determining the activity of an enzyme encoded by the xylose isomerase gene in the host cell.

102. The method of claim 101, wherein host cell has been genetically modified to produce, the low aldose reductase activity and the low xylitol dehydrogenase activity and genetically modified to produce overexpressed xylulokinase enzyme activity.

103. The method of claim 101 or 102, wherein the xylose isomerase gene is native to an anerobic fungi.

104. The method of claim 101 or claim 102, wherein the xylose isomerase gene is native to a bacteria.

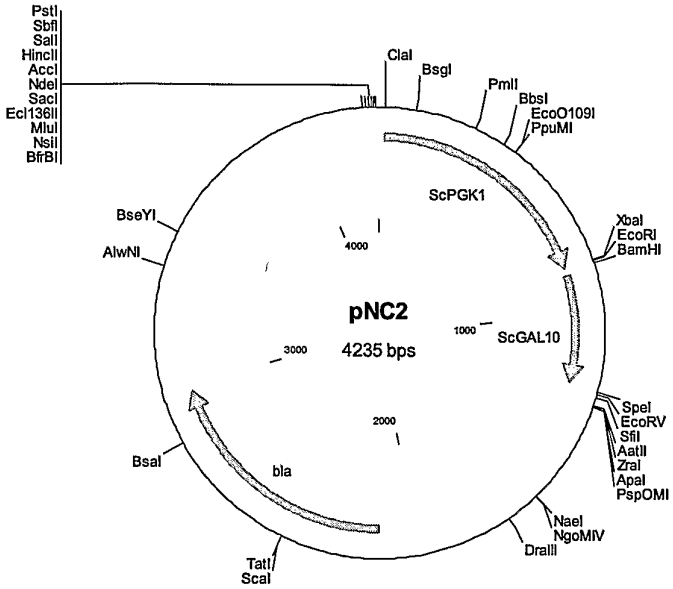
105. The method of any of claims 101-104, wherein the host cell is *K. marxianus*.

106. A yeast cell containing an exogenous yeast cell identified in accordance with any of claims 101-106.

107. A yeast cell having an exogenous xylose isomerase gene that is at least 80% homologous to SEQ. ID. NO. 151 but no more than 95% homologous to SEQ. ID. NO. 58.

108. A yeast cell having an exogenous xylose isomerase gene that produces an enzyme that is at least 80% homologous to SEQ. ID. NO. 152 but no more than 95% homologous to SEQ. ID. NO. 59.

Fig. 1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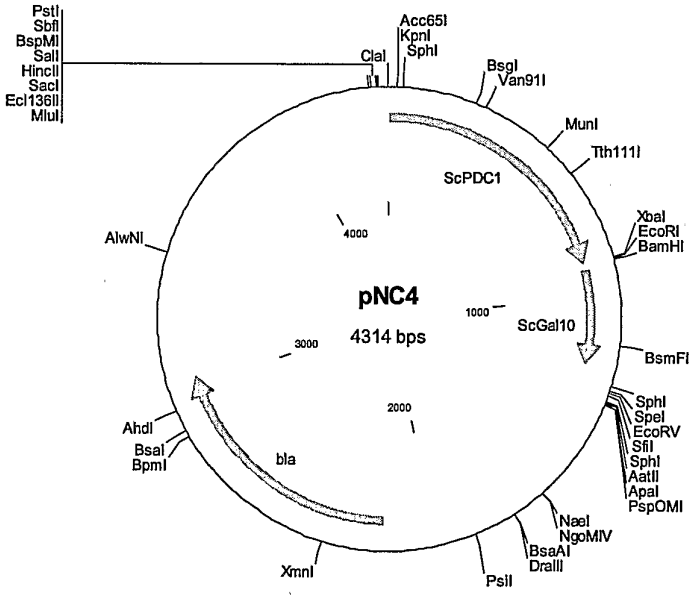
SalI
HincII
AccI
NdeI
SacI
Ecl136II
MluI
NsiI
BfrBI
ClaI
BsgI
PmlI
BbsI
EcoO109I
PpuMI
ScPGK1
XbaI
EcoRI
BamHI
BseYI
AlwNI
4000
pNC2
4235 bps
1000
ScGAL10
3000
2000
SpeI
EcoRV
SfiI
AatII
ZraI
ApaI
PspOMI
BsaI
bla
NaeI
NgoMIV
DraIII
TatI
ScaI

Fig. 2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SbfI
BspMI
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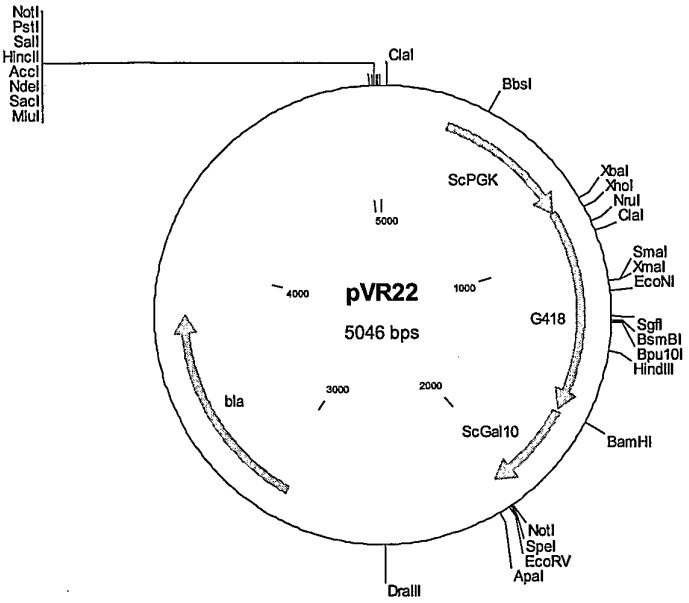
MluI
Acc65I
KpnI
SphI
ClaI
BsgI
Van91I
MunI
Tth111I
ScPDC1
XbaI
EcoRI
BamHI
AlwNI
4000
pNC4
4314 bps
1000
ScGal10
3000
BsmFI
2000
SphI
SpeI
EcoRV
SfiI
SphI
AatII
ApaI
PspOMI
AhdI
BsaI
BpmI
bla
NaeI
NgoMIV
BsaAI
DraIII
XmnI
PsiI

Fig. 3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AccI
NdeI
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ClaI
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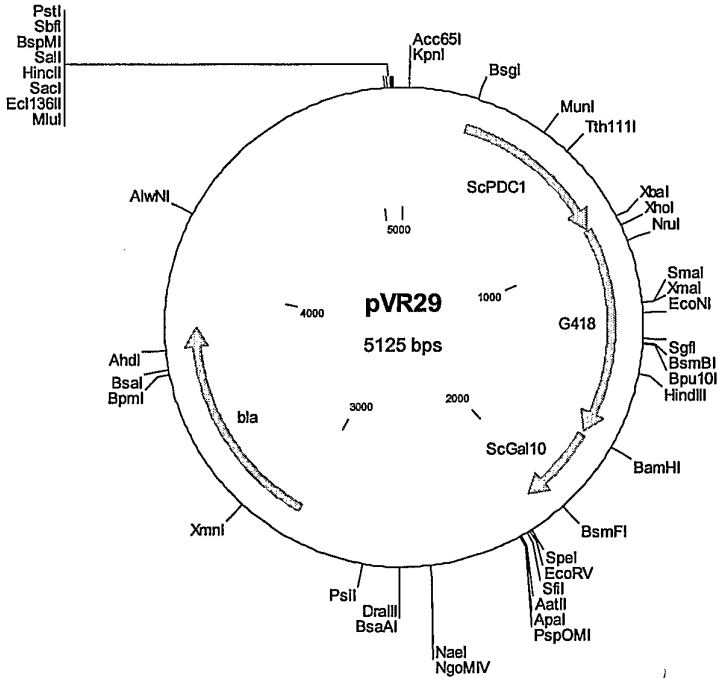
XhoI
NruI
ClaI
SmaI
XmaI
EcoNI
SgfI
BsmBI
Bpu10I
HindIII
BamHI
NotI
SpeI
EcoRV
ApaI
DraIII
bla
ScGal10
G418
pVR22
5046 bps
1000
2000
3000
4000
5000

Fig. 4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MunI
Tth111I
ScPDC1
XbaI
XhoI
NruI
SmaI
XmaI
EcoNI
SgfI
BsmBI
Bpu10I
HindIII
BamHI
BsmFI
SpeI
EcoRV
SfiI
AatII
ApaI
PspOMI
NaeI
NgoMIV
PsiI
DraIII
BsaAI
XmnI
AlwNI
AhdI
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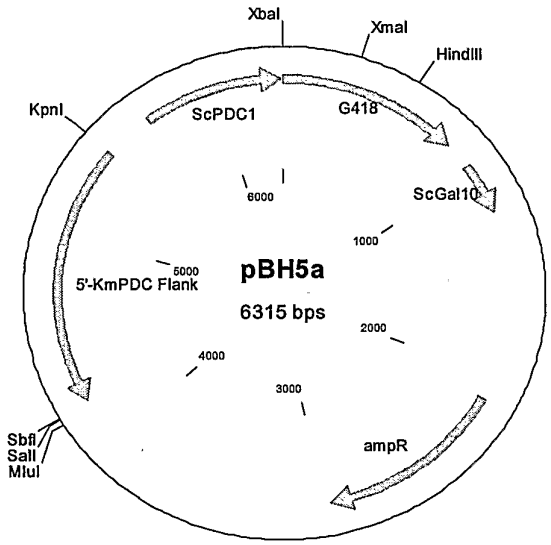
BpmI
bla
ScGal10
G418
pVR29
5125 bps
1000
2000
3000
4000
5000

Fig. 5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XmaI
HindIII
KpnI
ScPDC1
G418
ScGal10
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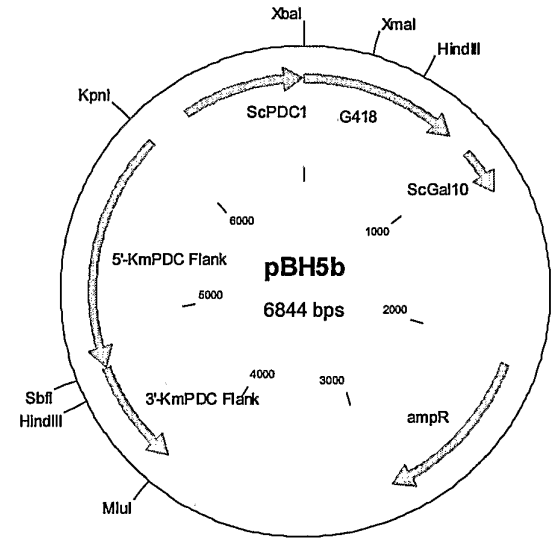
1000
5000
5'-KmPDC Flank
pBH5a
6315 bps
2000
4000
3000
SbfI
SalI
MluI
ampR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ScPDC1
G418
ScGal10
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1000
5'-KmPDC Flank
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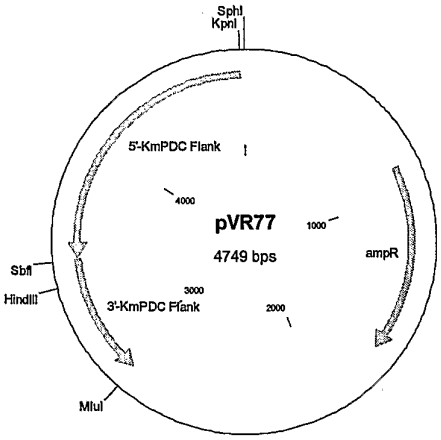
6844 bps
2000
4000
3000
SbfI
HindIII
3'-KmPDC Flank
ampR
MluI

Fig. 6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pVR77
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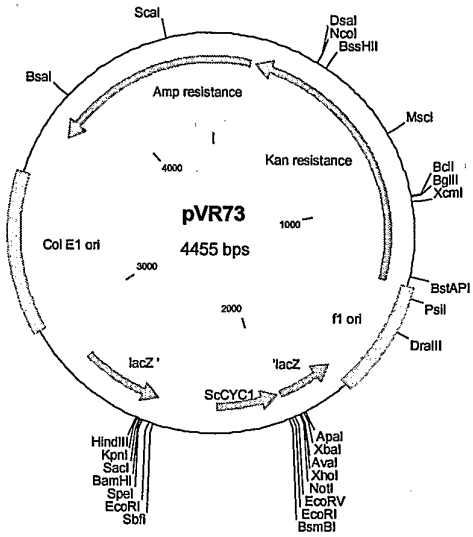
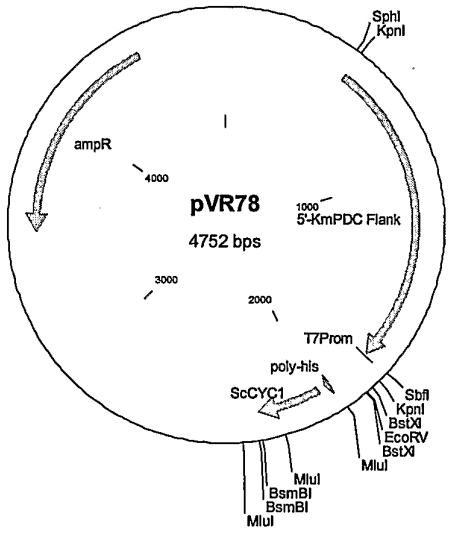
1000
ampR
SbfI
HindIII
3000
3'-KmPDC Flank
2000
MluI


ScaI
DsaI
NcoI
BssHII
BsaI
Amp resistance
MscI
4000
Kan resistance
BclI
BglII
XcmI
pVR73
1000
Col E1 ori
4455 bps
3000
BstAPI
PsiI
2000
f1 ori
DraIII
lacZ'
'lacZ
ScCYC1
HindIII
KpnI
SacI
BamHI
SpeI
EcoRI
SbfI
ApaI
XbaI
AvaI
XhoI
NotI
EcoRV
EcoRI
BsmBI


SphI
KpnI
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5'-KmPDC Flank
4752 bps
3000
2000
T7Prom
poly-his
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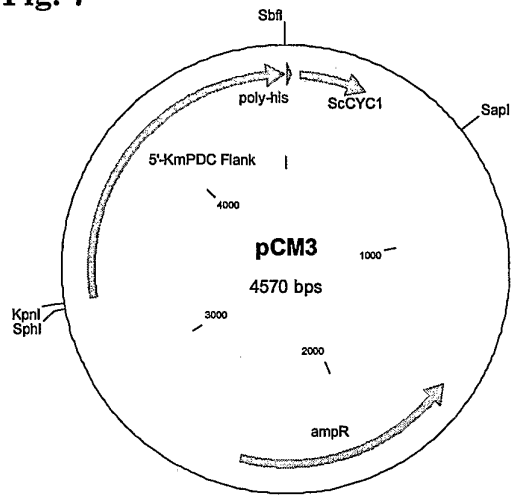
KpnI
BstXI
EcoRV
BstXI
MluI
MluI
BsmBI
BsmBI
MluI

Fig. 7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poly-his
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SapI
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pCM3
1000
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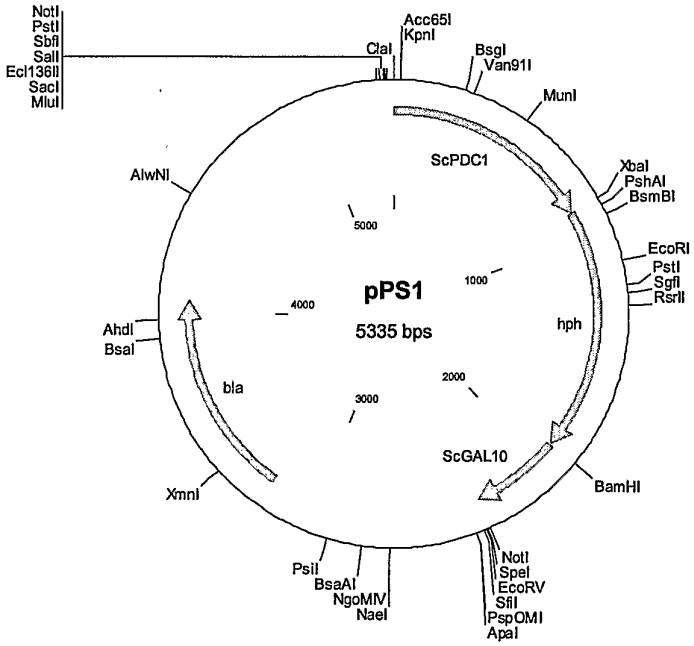
3000
KpnI
SphI
2000
ampR

Fig. 8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SalI
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Acc65I
KpnI
ClaI
BsgI
Van91I
MunI
ScPDC1
XbaI
PshAI
BsmBI
AlwNI
5000
EcoRI
PstI
SgfI
RsrII
1000
pPS1
4000
5335 bps
hph
AhdI
BsaI
2000
bla
3000
ScGAL10
BamHI
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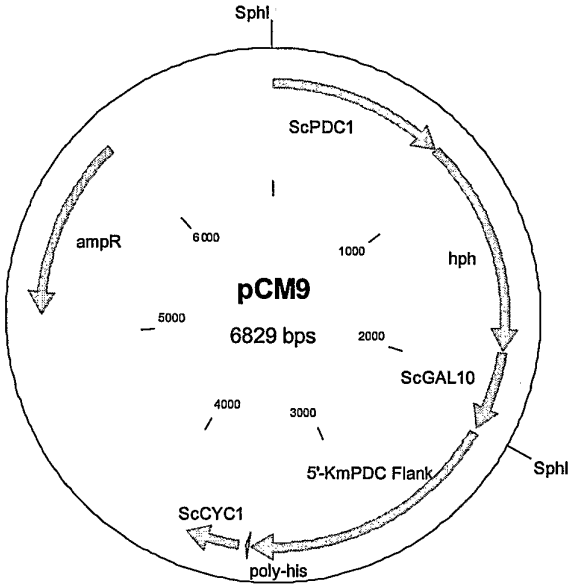
NgoMIV
NaeI
NotI
SpeI
EcoRV
SfiI
PspOMI
ApaI

Fig. 9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hph
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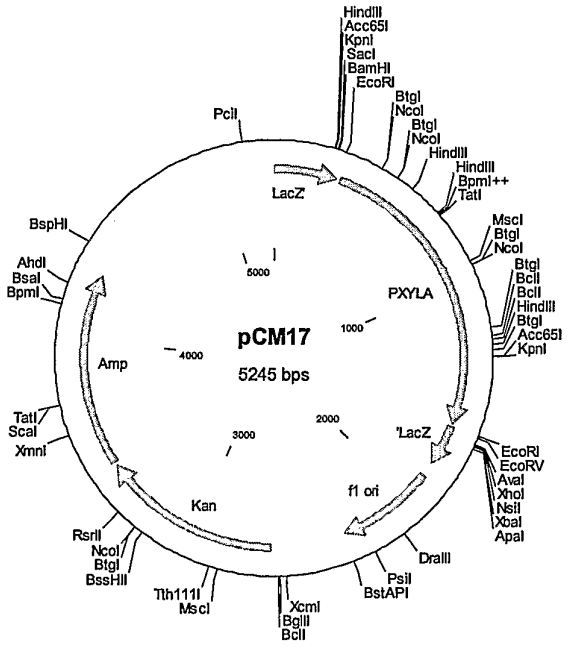
5'-KmPDC Flank
SphI
ScCYC1
poly-his

Fig. 10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NcoI
BtgI
NcoI
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TatI
PciI
LacZ'
MscI
BtgI
NcoI
BspHI
AhdI
BsaI
BpmI
5000
PXYLA
BtgI
BclI
BclI
HindIII
BtgI
Acc65I
KpnI
pCM17
1000
4000
Amp
5245 bps
TatI
ScaI
XmnI
2000
3000
'LacZ
EcoRI
EcoRV
AvaI
XhoI
NsiI
XbaI
ApaI
f1 ori
Kan
RsrII
NcoI
BtgI
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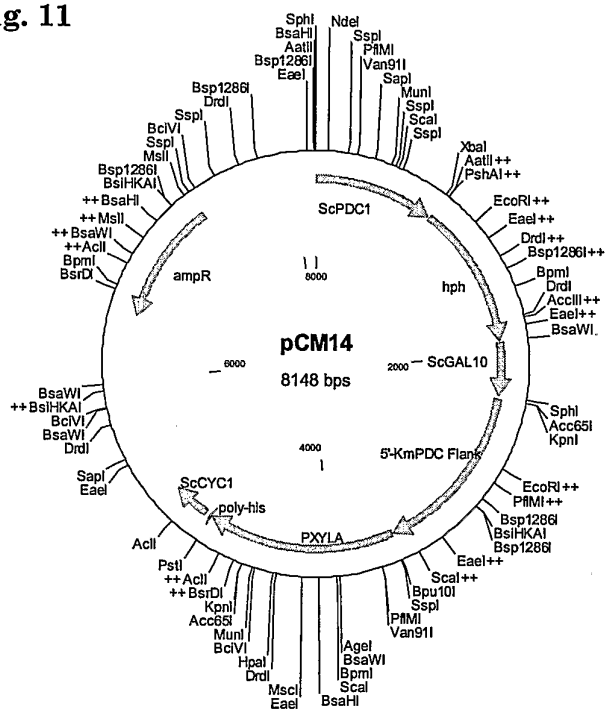
DraIII
PsiI
BstAPI
Tth111I
MscI
XcmI
BglII
BclI

Fig. 11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hph
ScGAL10
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ampR
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NdeI
SspI
PflMI
Van91I
SapI
MunI
SspI
ScaI
SspI
XbaI
AatII++
PshAI++
EcoRI++
EaeI++
DrdI++
Bsp1286I++
BpmI
DrdI
AccIII++
EaeI++
BsaWI
SphI
Acc65I
KpnI
EcoRI++
PflMI++
Bsp1286I
BsiHKAI
Bsp1286I
EaeI++
ScaI++
Bpu10I
SspI
PflMI
Van91I
AgeI
BsaWI
BpmI
ScaI
BsaHI
MscI
EaeI
HpaI
DrdI
BclVI
MunI
KpnI
Acc65I
++BsrDI
++AclI
PstI
AclI
SapI
EaeI
BsaWI
++BsiHKAI
BclVI
BsaWI
DrdI
BpmI
BsrDI
++AclI
++BsaWI
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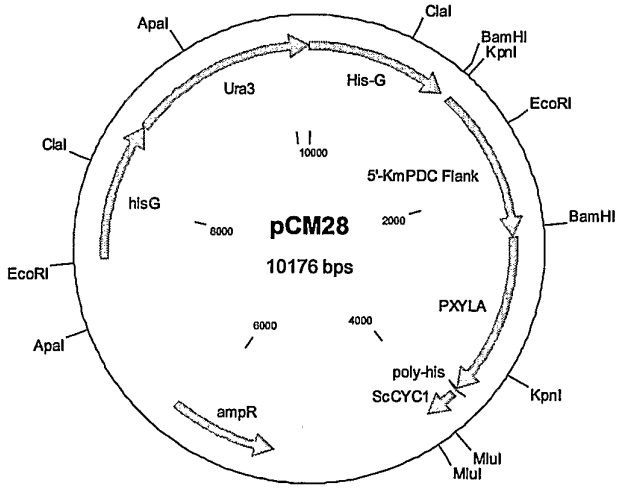
DrdI
Bsp1286I
EaeI

Fig. 12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EcoRI
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MluI
MluI
EcoRI
ApaI
ClaI

Fig. 13

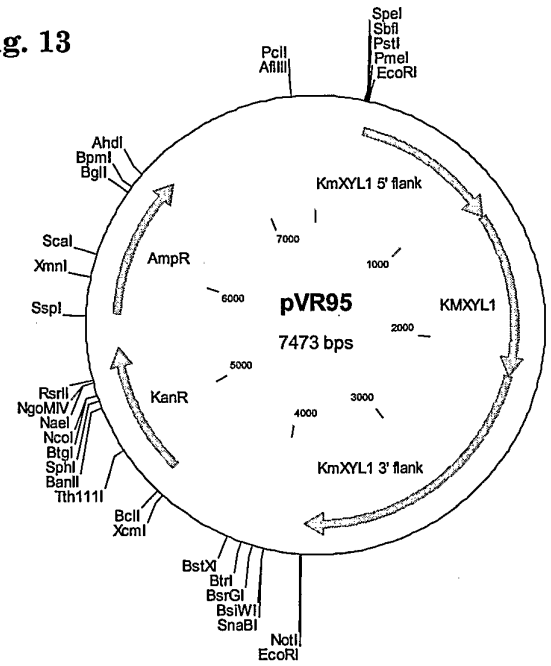


Fig. 14a

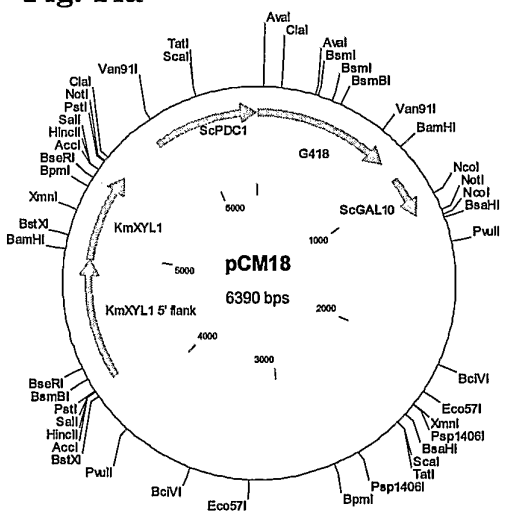


Fig. 14b

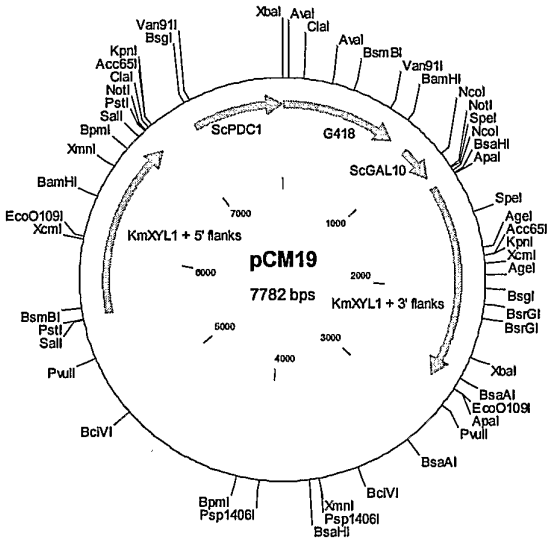
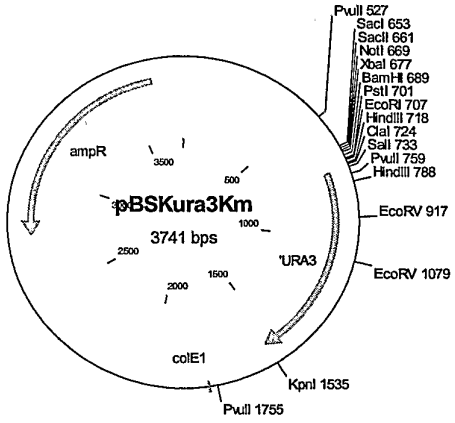


Fig. 15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SacII 661
NotI 669
XbaI 677
BamHI 689
PstI 701
EcoRI 707
HindIII 718
ClaI 724
SalI 733
PvuII 759
HindIII 788
EcoRV 917
EcoRV 1079
KpnI 1535
PvuII 1755
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pBSKura3Km
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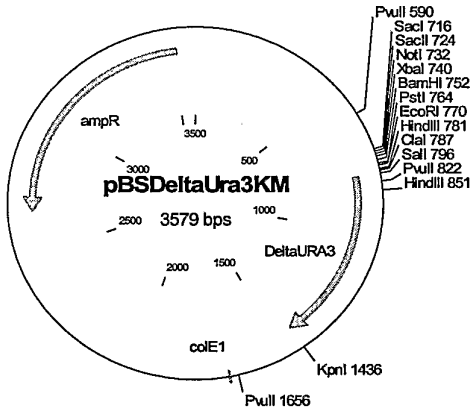
1000
2500
'URA3
1500
2000
colE1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SacII 724
NotI 732
XbaI 740
BamHI 752
PstI 764
EcoRI 770
HindIII 781
ClaI 787
SalI 796
PvuII 822
HindIII 851
KpnI 1436
PvuII 1656
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DeltaURA3
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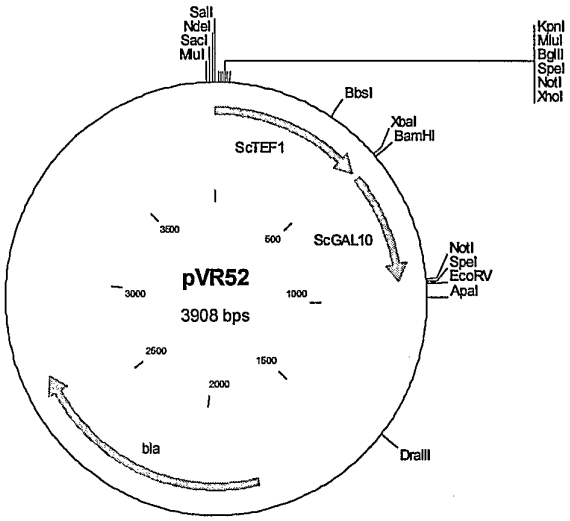
1500
colE1

Fig. 16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SacI
MluI
KpnI
MluI
BglII
SpeI
NotI
XhoI
BbsI
XbaI
BamHI
ScTEF1
ScGAL10
NotI
SpeI
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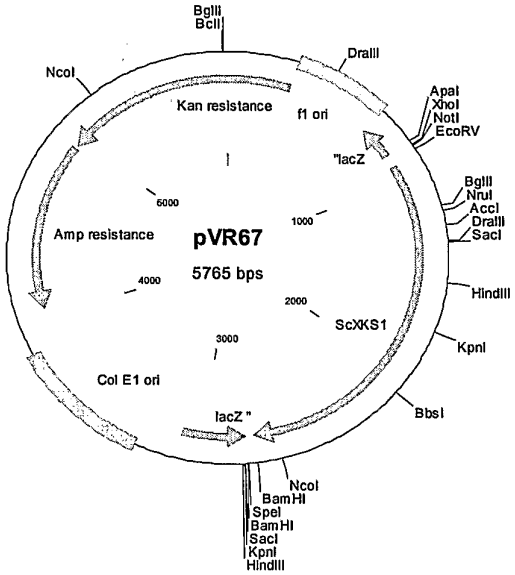
ApaI
pVR52
3908 bps
bla
DraIII
3500
600
1000
1500
2000
2500
3000


BglII
BclI
DraIII
NcoI
Kan resistance
f1 ori
ApaI
XhoI
NotI
EcoRV
"lacZ
BglII
NruI
AccI
DraIII
SacI
HindIII
KpnI
BbsI
NcoI
BamHI
SpeI
BamHI
SacI
KpnI
HindIII
Amp resistance
pVR67
5765 bps
Col E1 ori
lacZ"
ScXKS1
1000
2000
3000
4000
6000

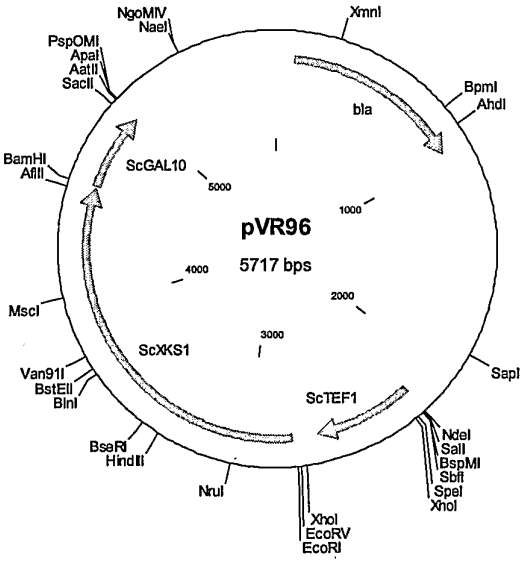

NgoMIV
NaeI
XmnI
PspOMI
ApaI
AatII
SacII
BpmI
AhdI
bla
BamHI
AflII
ScGAL10
pVR96
5717 bps
MscI
ScXKS1
SapI
Van91I
BstEII
BlnI
ScTEF1
NdeI
SalI
BspMI
SbfI
SpeI
XhoI
BseRI
HindII
NruI
XhoI
EcoRV
EcoRI
1000
2000
3000
4000
5000

Fig. 17

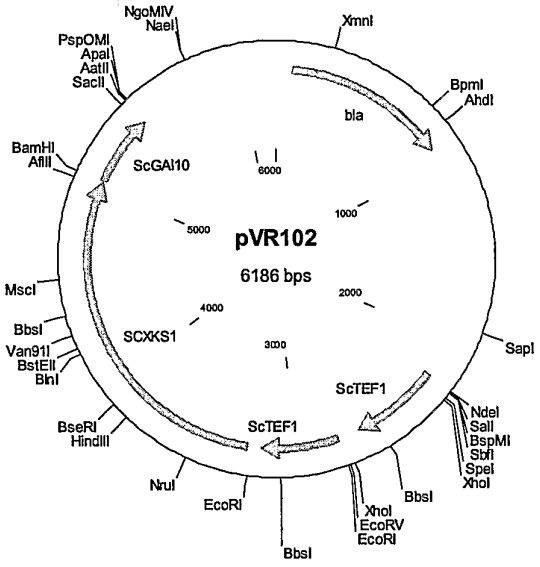

NgoMIV
NaeI
XmnI
PspOMI
ApaI
AatII
SacII
BpmI
AhdI
bla
BamHI
AflII
ScGAl10
6000
1000
5000
pVR102
6186 bps
MscI
2000
4000
BbsI
SCXKS1
3000
Van91I
BstEII
BlnI
SapI
ScTEF1
NdeI
SalI
BspMI
SbfI
SpeI
XhoI
BseRI
HindIII
ScTEF1
NruI
EcoRI
BbsI
XhoI
EcoRV
EcoRI
BbsI

Fig. 18

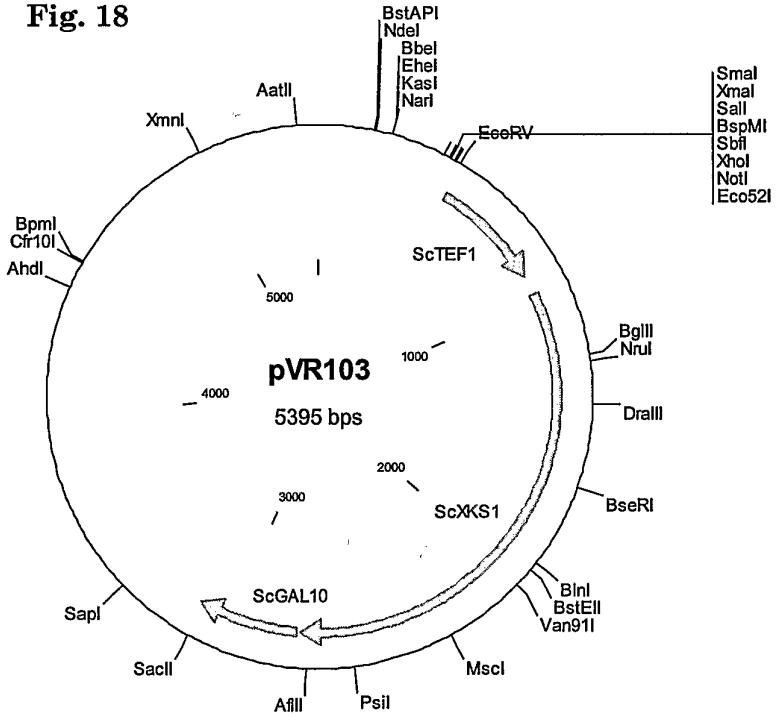
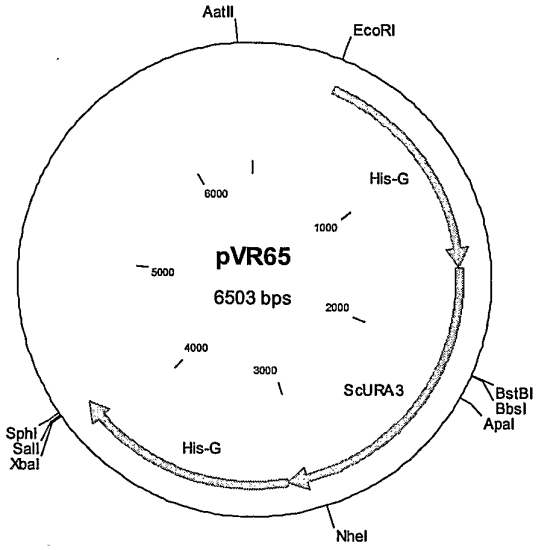

BstAPI
NdeI
BbeI
EheI
KasI
NarI
AatII
XmnI
SmaI
XmaI
SalI
BspMI
SbfI
XhoI
NotI
Eco52I
EcoRV
BpmI
Cfr10I
AhdI
ScTEF1
5000
BglII
NruI
1000
pVR103
4000
5395 bps
DraIII
2000
3000
ScXKS1
BseRI
BlnI
BstEII
Van91I
ScGAL10
SapI
MscI
SacII
AflII
PsiI

Fig. 19


AatII
EcoRI
His-G
6000
1000
pVR65
6503 bps
5000
2000
4000
3000
ScURA3
BstBI
BbsI
ApaI
SphI
SalI
XbaI
His-G
NheI

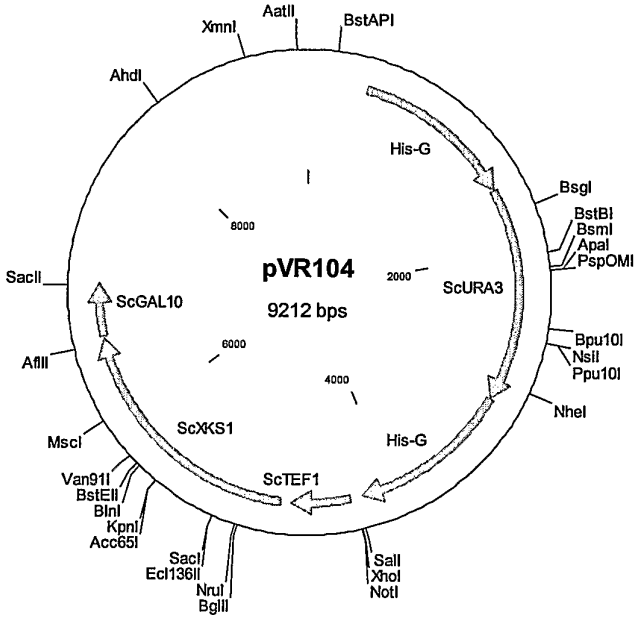
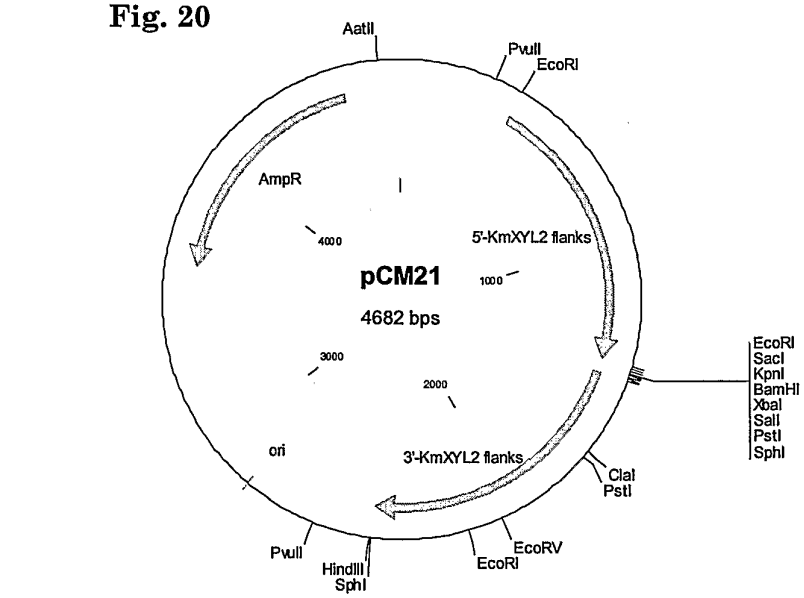

Xmnl
AatII
BstAPI
AhdI
His-G
BsgI
BstBI
BsmI
ApaI
PspOMI
6000
pVR104
2000
ScURA3
SacII
ScGAL10
9212 bps
Bpu10I
NsiI
Ppu10I
6000
AflII
4000
NheI
ScXKS1
MscI
His-G
Van91I
BstEII
BlnI
KpnI
Acc65I
ScTEF1
SacI
Ecl136II
NruI
BglII
SalI
XhoI
NotI

Fig. 20


AatII
PvuII
EcoRI
AmpR
4000
5'-KmXYL2 flanks
pCM21
1000
4682 bps
3000
2000
EcoRI
SacI
KpnI
BamHI
XbaI
SalI
PstI
SphI
ori
3'-KmXYL2 flanks
ClaI
PstI
PvuII
HindIII
SphI
EcoRV
EcoRI

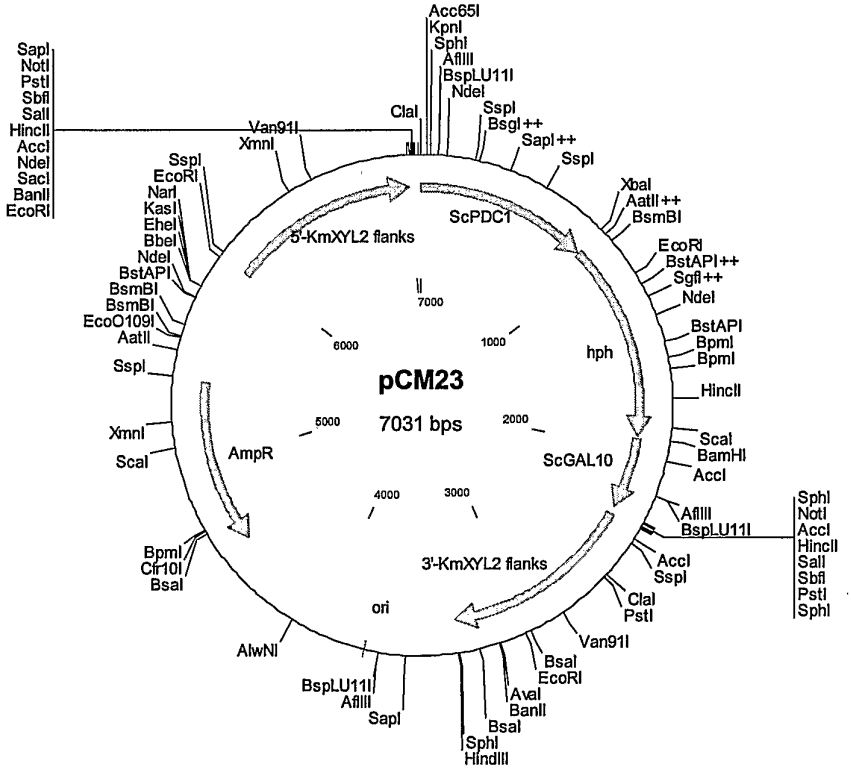

Acc65I
KpnI
SphI
AflIII
BspLU11I
NdeI
SspI
BsgI++
SapI++
SspI
XbaI
AatII++
BsmBI
EcoRI
BstAPI++
SgfI++
NdeI
BstAPI
BpmI
BpmI
HincII
ScaI
BamHI
AccI
SphI
NotI
AccI
HincII
SalI
SbfI
PstI
SphI
AflIII
BspLU11I
AccI
SspI
ClaI
PstI
Van91I
BsaI
EcoRI
AvaI
BanII
BsaI
SphI
HindIII
SapI
BspLU11I
AflIII
AlwNI
ori
3'-KmXYL2 flanks
BpmI
Ctr10I
BsaI
ScaI
XmnI
AmpR
5000
7000
6000
1000
pCM23
7031 bps
2000
4000
3000
hph
ScGAL10
ScPDC1
5'-KmXYL2 flanks
SapI
NotI
PstI
SbfI
SalI
HincII
AccI
NdeI
SacI
BanII
EcoRI
Van91I
XmnI
ClaI
SspI
EcoRI
NarI
KasI
EheI
BbeI
NdeI
BstAPI
BsmBI
BsmBI
EcoO109I
AatII
SspI

Fig. 21

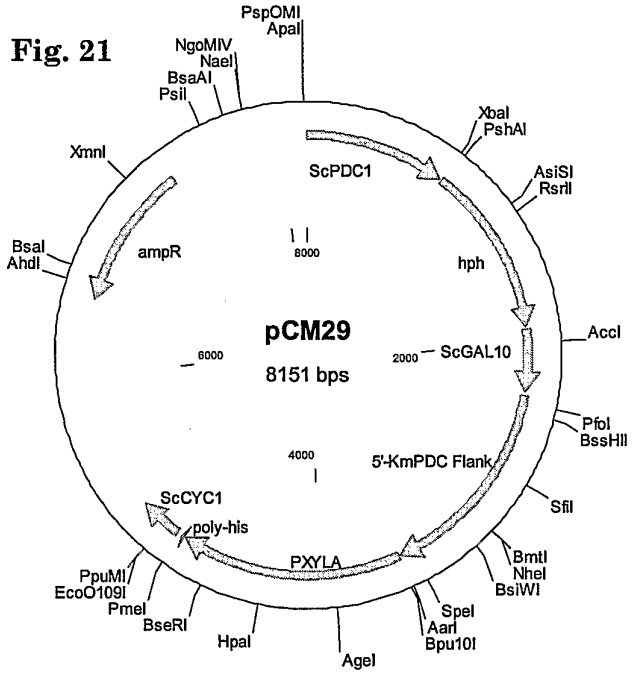

PspOMI
ApaI
NgoMIV
NaeI
BsaAI
PsiI
XbaI
PshAI
AsiSI
RsrII
XmnI
ScPDC1
BsaI
AhdI
ampR
8000
hph
pCM29
AccI
6000
2000
ScGAL10
8151 bps
PfoI
BssHII
4000
5'-KmPDC Flank
ScCYC1
SfiI
poly-his
BmtI
NheI
PXYLA
BsiWI
PpuMI
EcoO109I
PmeI
SpeI
AarI
BseRI
Bpu10I
HpaI
AgeI

Fig. 22

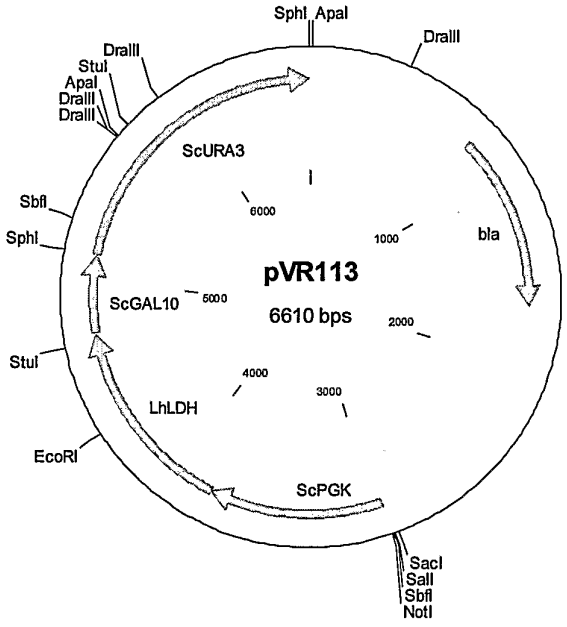

SphI
ApaI
DraIII
DraIII
StuI
ApaI
DraIII
DraIII
ScURA3
SbfI
6000
SphI
1000
bla
pVR113
ScGAL10
5000
6610 bps
2000
StuI
4000
3000
LhLDH
EcoRI
ScPGK
SacI
SalI
SbfI
NotI

Fig. 23

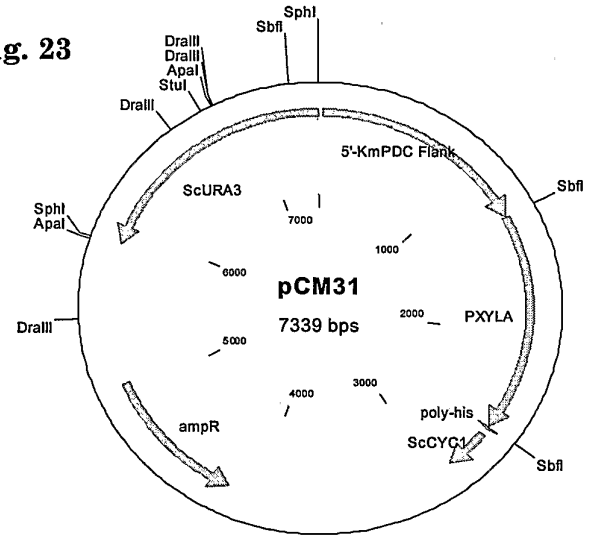

SphI
SbfI
DraIII
DraIII
ApaI
StuI
DraIII
5'-KmPDC Flank
SbfI
ScURA3
SphI
ApaI
7000
1000
6000
pCM31
7339 bps
2000
PXYLA
DraIII
5000
3000
4000
poly-his
ampR
ScCYC1
SbfI

Fig. 24

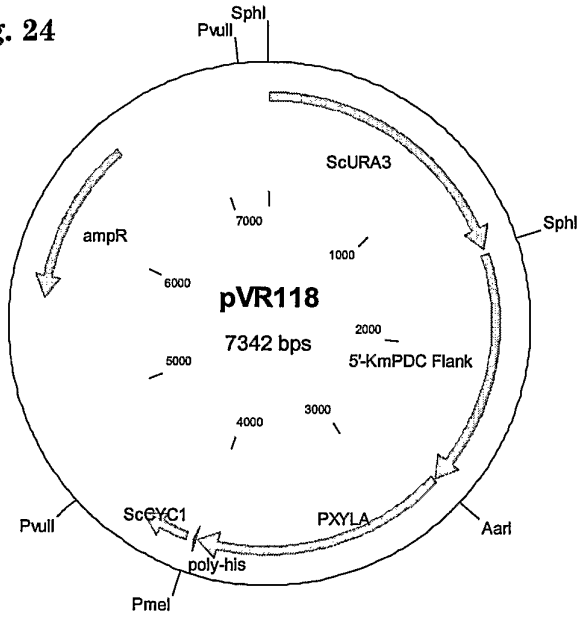

SphI
PvuII
ScURA3
SphI
ampR
7000
1000
6000
pVR118
2000
7342 bps
5000
5'-KmPDC Flank
3000
4000
PvuII
ScCYC1
PXYLA
AarI
poly-his
PmeI

Fig. 25

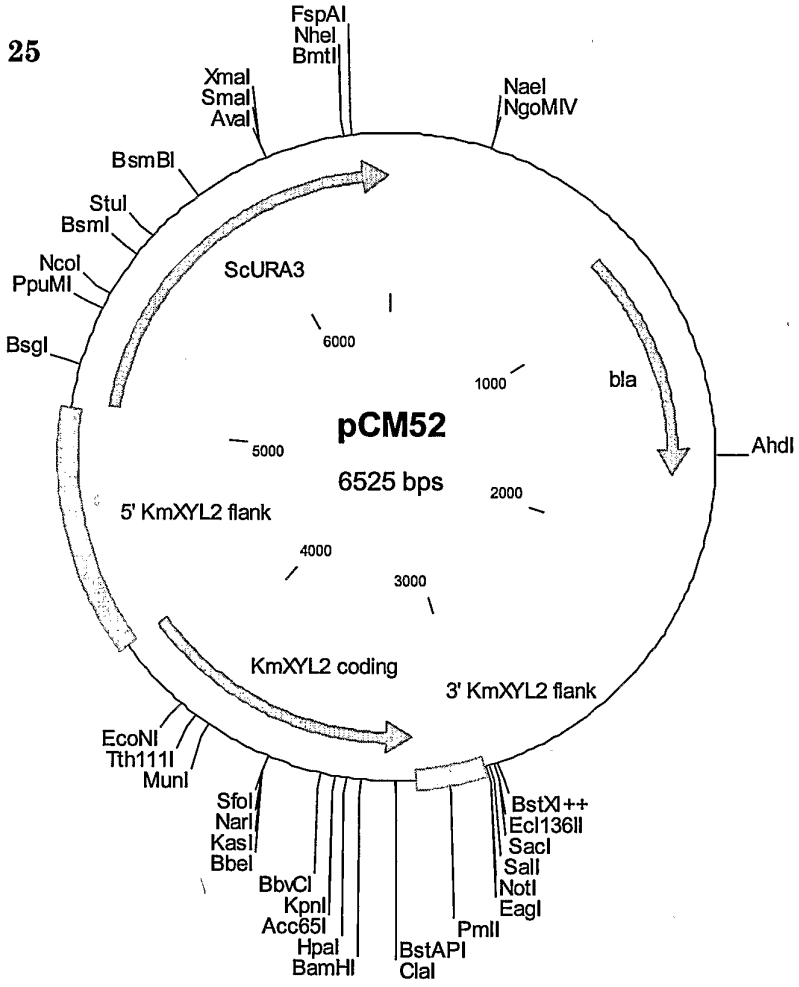

FspAI
NheI
BmtI
XmaI
SmaI
AvaI
NaeI
NgoMIV
BsmBI
StuI
BsmI
NcoI
PpuMI
BsgI
ScURA3
6000
1000
bla
pCM52
6525 bps
5000
2000
AhdI
5' KmXYL2 flank
4000
3000
KmXYL2 coding
3' KmXYL2 flank
EcoNI
Tth111I
MunI
SfoI
NarI
KasI
BbeI
BbvCI
KpnI
Acc65I
HpaI
BamHI
BstAPI
ClaI
PmlI
BstXI++
Ecl136II
SacI
SalI
NotI
EagI

Fig. 26

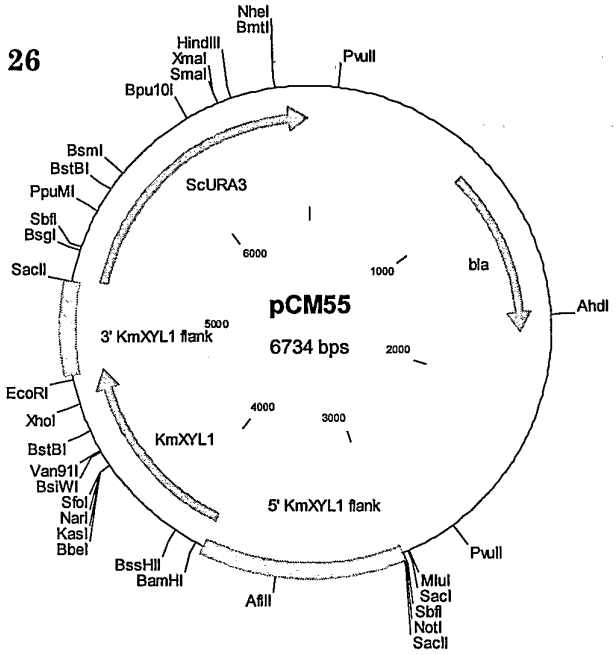

NheI
BmtI
HindIII
XmaI
SmaI
PvuII
Bpu10I
BsmI
BstBI
PpuMI
SbfI
BsgI
SacII
ScURA3
6000
1000
bla
AhdI
pCM55
6734 bps
5000
3' KmXYL1 flank
2000
EcoRI
XhoI
BstBI
Van91I
BsiWI
SfoI
NarI
KasI
BbeI
4000
3000
KmXYL1
5' KmXYL1 flank
PvuII
BssHII
BamHI
AflII
MluI
SacI
SbfI
NotI
SacII

Fig. 27

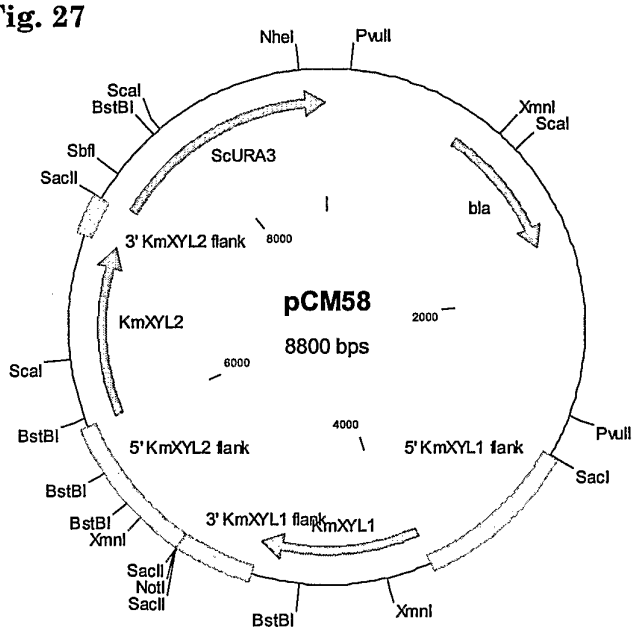

NheI
PvuII
ScaI
BstBI
XmnI
ScaI
SbfI
SacII
ScURA3
bla
3' KmXYL2 flank
8000
pCM58
2000
KmXYL2
8800 bps
6000
ScaI
BstBI
4000
PvuII
5' KmXYL2 flank
5' KmXYL1 flank
SacI
BstBI
BstBI
XmnI
3' KmXYL1 flank
KmXYL1
SacII
NotI
SacII
BstBI
XmnI

Fig. 28

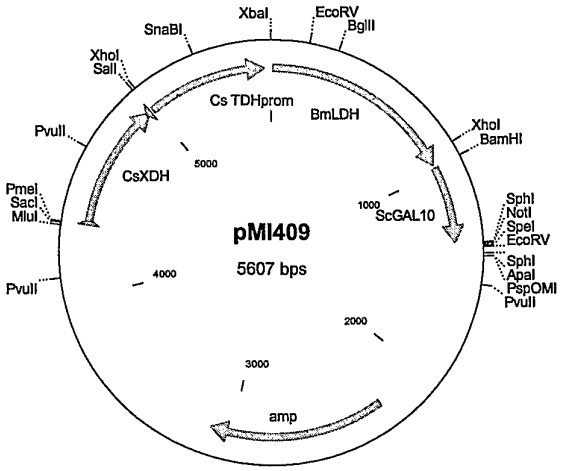

XbaI
EcoRV
BglII
SnaBI
XhoI
SalI
Cs TDHprom
BmLDH
XhoI
BamHI
PvuII
5000
CsXDH
PmeI
SacI
MluI
1000
ScGAL10
SphI
NotI
SpeI
EcoRV
pMI409
5607 bps
SphI
ApaI
PspOMI
PvuII
4000
PvuII
2000
3000
amp

Fig. 29

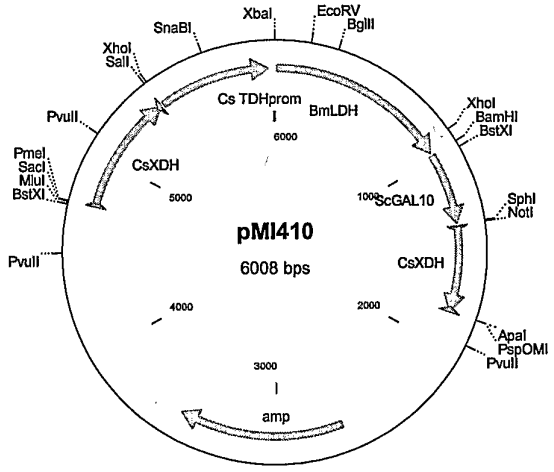

XbaI
EcoRV
BglII
SnaBI
XhoI
SalI
Cs TDHprom
BmLDH
XhoI
BamHI
BstXI
PvuII
6000
PmeI
SacI
MluI
BstXI
CsXDH
5000
1000
ScGAL10
SphI
NotI
pMI410
6008 bps
PvuII
CsXDH
4000
2000
ApaI
PspOMI
PvuII
3000
amp

Fig.30

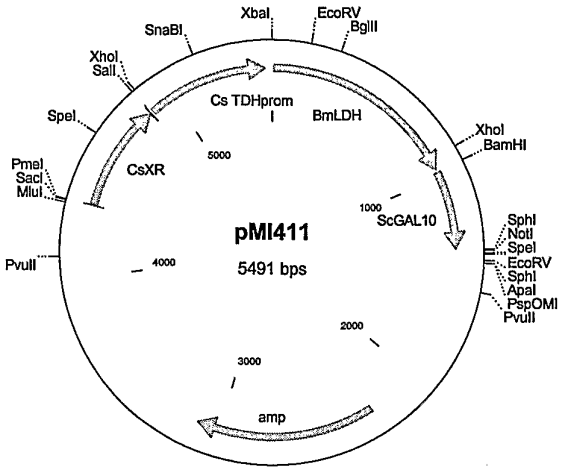

XbaI
EcoRV
BglII
SnaBI
XhoI
SalI
Cs TDHprom
BmLDH
SpeI
XhoI
BamHI
PmeI
SacI
MluI
CsXR
5000
1000
ScGAL10
SphI
NotI
SpeI
EcoRV
SphI
ApaI
PspOMI
PvuII
pMI411
5491 bps
PvuII
4000
2000
3000
amp

Fig. 31

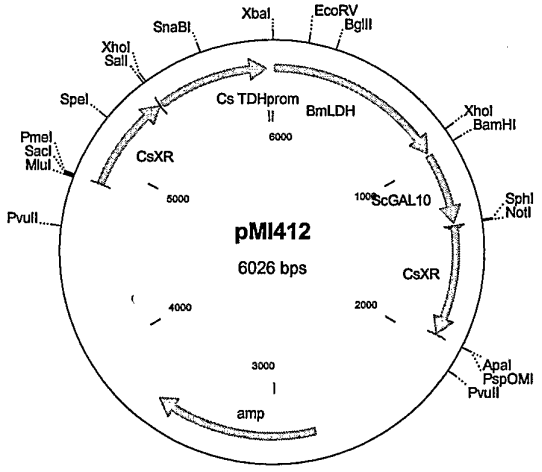

XbaI
EcoRV
BglII
SnaBI
XhoI
SalI
SpeI
Cs TDHprom
II
BmLDH
XhoI
BamHI
PmeI
SacI
MluI
6000
CsXR
5000
1000
ScGAL10
SphI
NotI
PvuII
pMI412
6026 bps
CsXR
4000
2000
ApaI
PspOMI
PvuII
3000
amp

Fig. 32

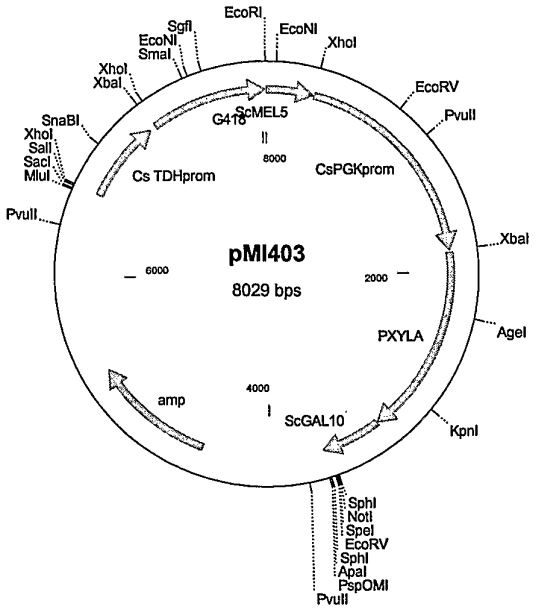

EcoRI
EcoNI
XhoI
SgfI
EcoNI
SmaI
XhoI
XbaI
EcoRV
PvuII
SnaBI
XhoI
SalI
SacI
MluI
G418
ScMEL5
8000
Cs TDHprom
CsPGKprom
PvuII
XbaI
pMI403
8029 bps
6000
2000
AgeI
PXYLA
4000
amp
ScGAL10
KpnI
SphI
NotI
SpeI
EcoRV
SphI
ApaI
PspOMI
PvuII

Fig. 33

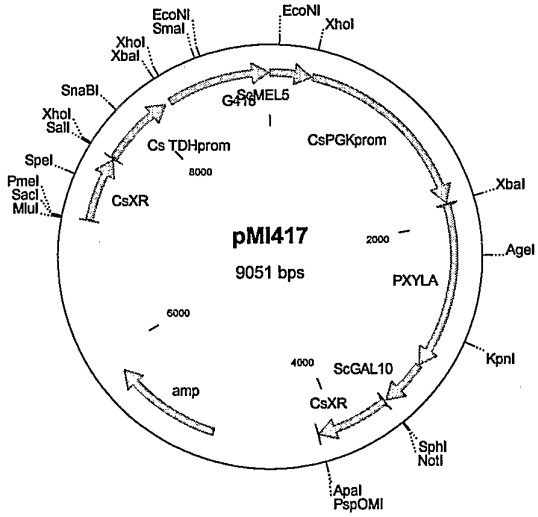

EcoNI
SmaI
EcoNI
XhoI
XhoI
XbaI
SnaBI
G418
ScMEL5
XhoI
SalI
Cs TDHprom
CsPGKprom
SpeI
8000
PmeI
SacI
MluI
CsXR
XbaI
pMI417
2000
AgeI
9051 bps
PXYLA
6000
4000
ScGAL10
KpnI
amp
CsXR
SphI
NotI
ApaI
PspOMI

Fig. 34

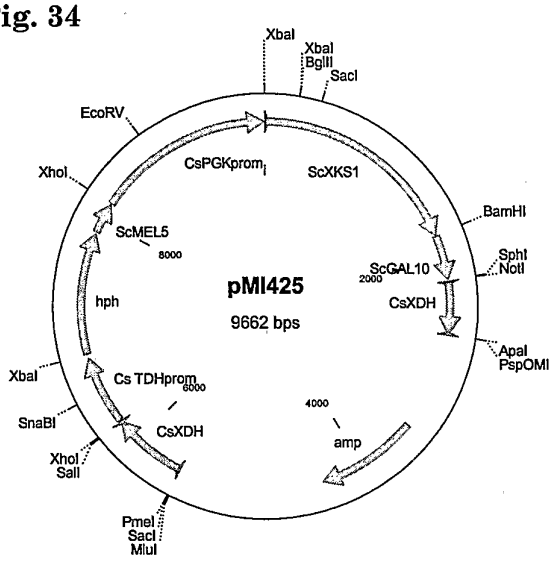

XbaI
XbaI
BglII
SacI
EcoRV
XhoI
CsPGKprom
ScXKS1
BamHI
ScMEL5
8000
SphI
NotI
ScGAL10
2000
pMI425
9662 bps
hph
CsXDH
ApaI
PspOMI
XbaI
Cs TDHprom
6000
4000
SnaBI
CsXDH
amp
XhoI
SalI
PmeI
SacI
MluI

Fig.35

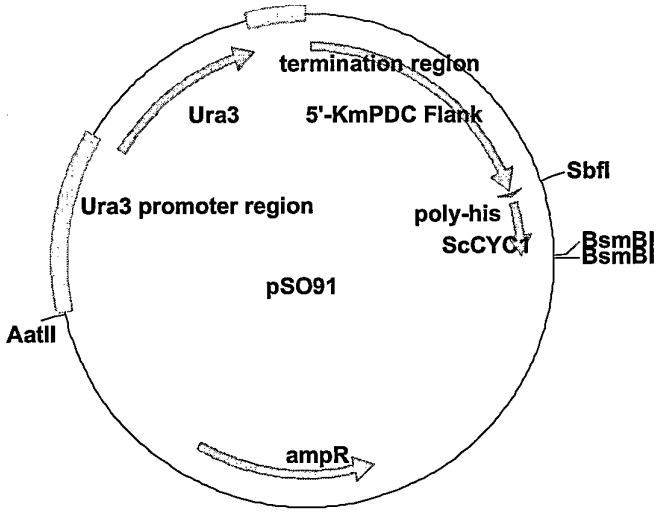

termination region
Ura3
5'-KmPDC Flank
SbfI
Ura3 promoter region
poly-his
ScCYC1
BsmBI
BsmBI
pSO91
AatII
ampR

Fig. 36

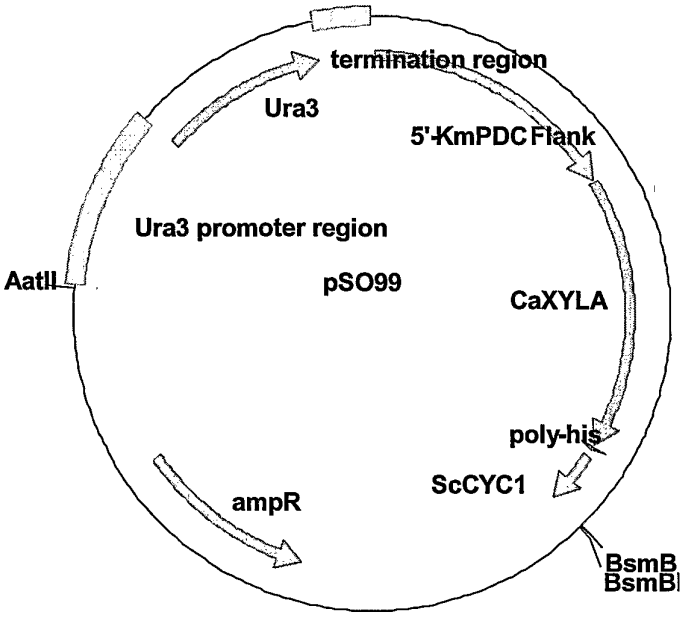

termination region
Ura3
5'-KmPDC Flank
Ura3 promoter region
AatII
pSO99
CaXYLA
poly-his
ScCYC1
ampR
BsmB
BsmBI

Fig. 37

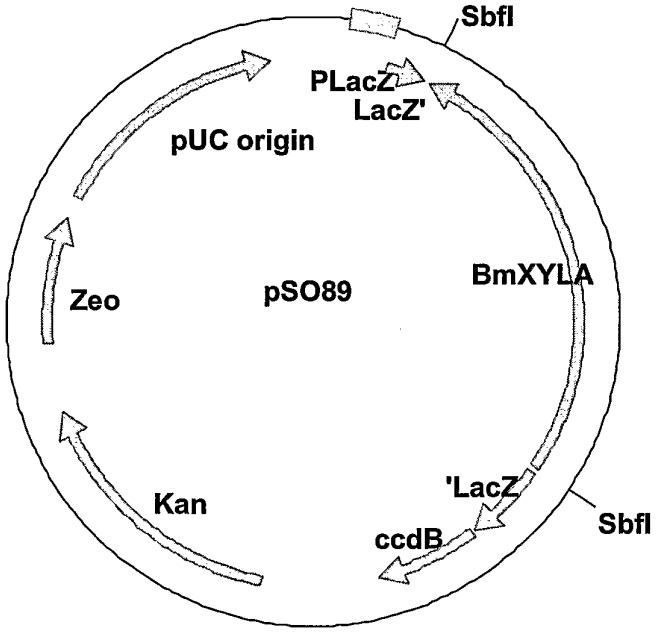

SbfI
PLacZ
LacZ'
pUC origin
BmXYLA
Zeo
pSO89
'LacZ
Kan
ccdB
SbfI

Fig. 38

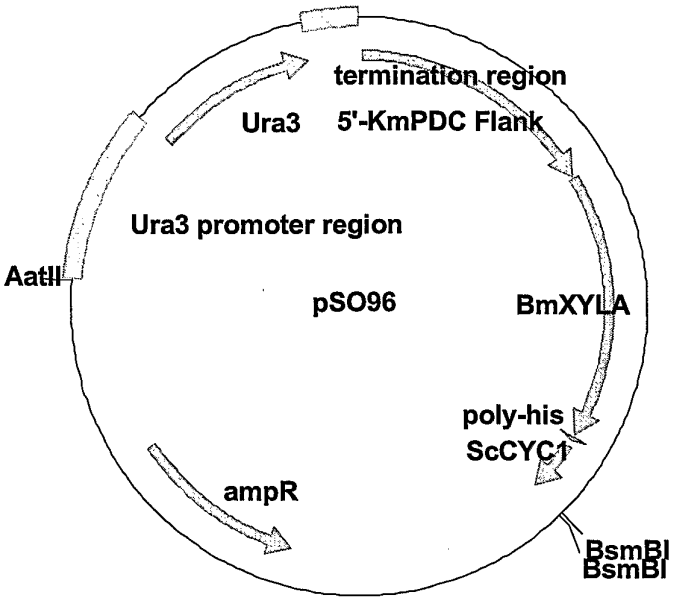

termination region
Ura3
5'-KmPDC Flank
Ura3 promoter region
AatII
pSO96
BmXYLA
poly-his
ScCYC1
ampR
BsmBI
BsmBI

Fig. 39

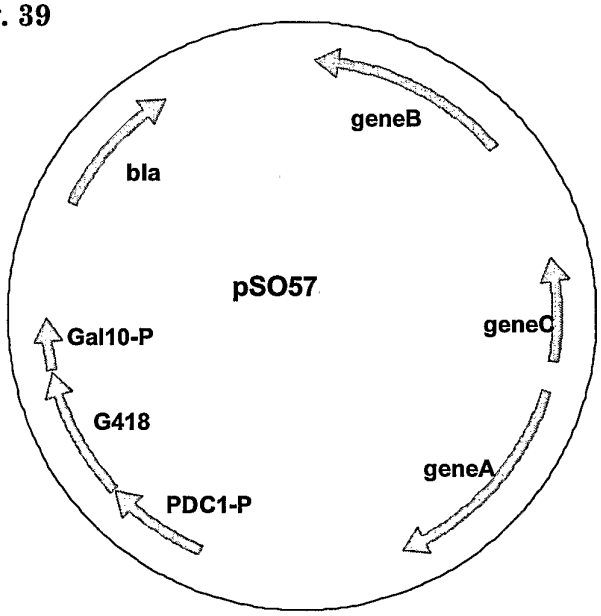
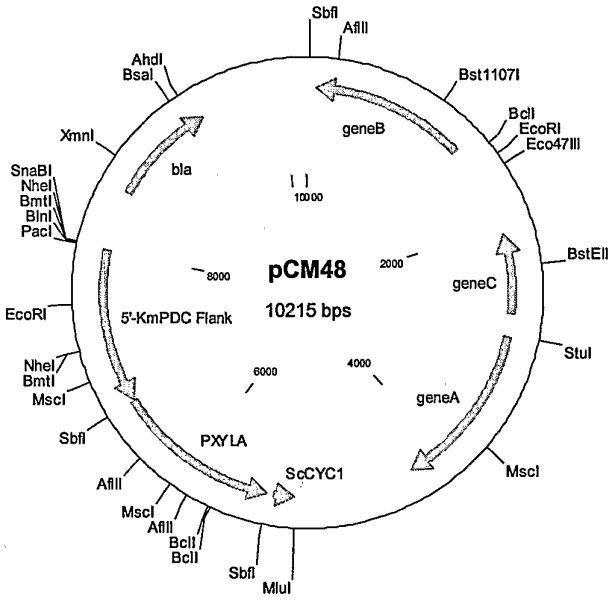

geneB
bla
pSO57
geneC
Gal10-P
G418
geneA
PDC1-P

Fig. 40


pCM48
10215 bps
SbfI
AflII
Bst1107I
BclI
EcoRI
Eco47III
BstEII
StuI
MscI
ScCYC1
PXYLA
5'-KmPDC Flank
EcoRI
NheI
BmtI
MscI
SbfI
AflII
MscI
AflII
BclI
BclI
SbfI
MluI
XmnI
AhdI
BsaI
SnaBI
NheI
BmtI
BlnI
PacI
bla
geneB
geneC
geneA
2000
4000
6000
8000
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1182A WO.ST25
SEQUENCE LISTING

<110> Cargill Dow LLC
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<120> Genetically Modified Yeast Species, and Fermentation Processes Using Genetically Modified Yeast

<130> 1182A WO

<150> US 60/467,727
<151> 2003-05-02

<160> 167

<170> PatentIn version 3.2

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aagcaaaagg ttgatgctgg tttcgaaatc atgcaaaagc ttggtattcc atactactgt 300

ttccacgatg ttgatcttgt ttccgaaggt aactctattg aagaatacga atccaacctt 360

aaggctgtcg ttgcttacct caaggaaaag caaaaggaaa ccggtattaa gcttctctgg 420

agtactgcta acgtcttcgg tcacaagcgt tacatgaacg gtgcctccac taacccagac 480

tttgatgttg tcgcccgtgc tattgttcaa attaagaacg ccatagacgc cggtattgaa 540

cttggtgctg aaaactacgt cttctggggt ggtcgtgaag gttacatgag tctccttaac 600

actgaccaaa agcgtgaaaa ggaacacatg gccactatgc ttaccatggc tcgtgactac 660

gctcgttcca agggattcaa gggtactttc ctcattgaac caaagccaat ggaaccaacc 720

aagcaccaat acgatgttga cactgaaacc gctattggtt tccttaaggc ccacaactta 780

gacaaggact tcaaggtcaa cattgaagtt aaccacgcta ctcttgctgg tcacactttc 840

gaacacgaac ttgcctgtgc tgttgatgct ggtatgctcg gttccattga tgctaaccgt 900

ggtgactacc aaaacggttg ggatactgat caattcccaa ttgatcaata cgaactcgtc 960

caagcttgga tggaaatcat ccgtggtggt ggtttcgtta ctggtggtac caacttcgat 1020

gccaagactc gtcgtaactc tactgacctc gaagacatca tcattgccca cgtttctggt 1080

atggatgcta tggctcgtgc tcttgaaaac gctgccaagc tcctccaaga atctccatac 1140

accaagatga agaaggaacg ttacgcttcc ttcgacagtg gtattggtaa ggactttgaa 1200

gatggtaagc tcaccctcga acaagtttac gaatacggta agaagaacgg tgaaccaaag 1260

caaacttctg gtaagcaaga actctacgaa gctattgttg ccatgtacca ataa 1314

<210> 59
<211> 437
<212> PRT
<213> Artificial

<220>
<223> Deduced amino acid sequence encoded by reconstructed Piromyces sp. E2 xylose isomerase gene

<400> 59

Met Ala Lys Glu Tyr Phe Pro Gln Ile Gln Lys Ile Lys Phe Glu Gly
1 5 10 15

Lys Asp Ser Lys Asn Pro Leu Ala Phe His Tyr Tyr Asp Ala Glu Lys
20 25 30

Glu Val Met Gly Lys Lys Met Lys Asp Trp Leu Arg Phe Ala Met Ala
35 40 45

Trp Trp His Thr Leu Cys Ala Glu Gly Ala Asp Gln Phe Gly Gly Gly
50 55 60

Thr Lys Ser Phe Pro Trp Asn Glu Gly Thr Asp Ala Ile Glu Ile Ala
65 70 75 80

Lys Gln Lys Val Asp Ala Gly Phe Glu Ile Met Gln Lys Leu Gly Ile
85 90 95

Pro Tyr Tyr Cys Phe His Asp Val Asp Leu Val Ser Glu Gly Asn Ser
100 105 110

Ile Glu Glu Tyr Glu Ser Asn Leu Lys Ala Val Val Ala Tyr Leu Lys
115 120 125

Glu Lys Gln Lys Glu Thr Gly Ile Lys Leu Leu Trp Ser Thr Ala Asn
130 135 140

Val Phe Gly His Lys Arg Tyr Met Asn Gly Ala Ser Thr Asn Pro Asp
145 150 155 160

Phe Asp Val Val Ala Arg Ala Ile Val Gln Ile Lys Asn Ala Ile Asp

165 170 175

Ala Gly Ile Glu Leu Gly Ala Glu Asn Tyr Val Phe Trp Gly Gly Arg
180 185 190

Glu Gly Tyr Met Ser Leu Leu Asn Thr Asp Gln Lys Arg Glu Lys Glu
195 200 205

His Met Ala Thr Met Leu Thr Met Ala Arg Asp Tyr Ala Arg Ser Lys
210 215 220

Gly Phe Lys Gly Thr Phe Leu Ile Glu Pro Lys Pro Met Glu Pro Thr
225 230 235 240

Lys His Gln Tyr Asp Val Asp Thr Glu Thr Ala Ile Gly Phe Leu Lys
245 250 255

Ala His Asn Leu Asp Lys Asp Phe Lys Val Asn Ile Glu Val Asn His
260 265 270

Ala Thr Leu Ala Gly His Thr Phe Glu His Glu Leu Ala Cys Ala Val
275 280 285

Asp Ala Gly Met Leu Gly Ser Ile Asp Ala Asn Arg Gly Asp Tyr Gln
290 295 300

Asn Gly Trp Asp Thr Asp Gln Phe Pro Ile Asp Gln Tyr Glu Leu Val
305 310 315 320

Gln Ala Trp Met Glu Ile Ile Arg Gly Gly Gly Phe Val Thr Gly Gly
325 330 335

Thr Asn Phe Asp Ala Lys Thr Arg Arg Asn Ser Thr Asp Leu Glu Asp
340 345 350

Ile Ile Ile Ala His Val Ser Gly Met Asp Ala Met Ala Arg Ala Leu
355 360 365

Glu Asn Ala Ala Lys Leu Leu Gln Glu Ser Pro Tyr Thr Lys Met Lys
370 375 380

Lys Glu Arg Tyr Ala Ser Phe Asp Ser Gly Ile Gly Lys Asp Phe Glu
385 390 395 400

Asp Gly Lys Leu Thr Leu Glu Gln Val Tyr Glu Tyr Gly Lys Lys Asn
405 410 415

Gly Glu Pro Lys Gln Thr Ser Gly Lys Gln Glu Leu Tyr Glu Ala Ile
420 425 430

Val Ala Met Tyr Gln

<210> 60
<211> 42
<212> DNA
<213> Artificial

<220>
<223> Piromyces xylose isomerase gene amplification primer

<400> 60
atcgtattcc tgcaggatgg ctaaggaata tttcccacaa at 42

<210> 61
<211> 44
<212> DNA
<213> Artificial

<220>
<223> Piromyces xylose isomerase amplification primer

<400> 61
atatcgaacc tgcaggttat tggtacatgg caacaatagc ttcg 44

<210> 62
<211> 43
<212> DNA
<213> Artificial

<220>
<223> Piromyces xylose isomerase gene mutagenic primer

<400> 62
gctattgttg ccatgtacca acctgcagga catcatcacc atc 43

<210> 63
<211> 26
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for upstream flank of K. marxianus xylose reductase gene

<400> 63
aggtaatata ggtaaacaaa gatcac 26

<210> 64
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for upstream flank of K. marxianus xylose reductase gene

<400> 64
tatgtatgtg tgtgctactt accacag 27

<210> 65
<211> 28
<212> DNA

<213> Artificial

<220>
<223> Amplification primer for downstream flank of K. marxianus xylose reductase gene

<400> 65
cctggaattt tcatgaaact gatataag 28

<210> 66
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for downstream flank of K. marxianus xylose reductase gene

<400> 66
actaaactcg ctttgttctg gctcatc 27

<210> 67
<211> 25
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for K. marxianus xylose reductase region

<400> 67
gccagaggta gagagcacaa agtaa 25

<210> 68
<211> 25
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for K. marxianus xylose reductase region

<400> 68
cgaagccaac tcgcttctat ctggt 25

<210> 69
<211> 31
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for K. marxianus promoter region.

<400> 69
tattactgca gtgcacccga aaagtttgag a 31

<210> 70
<211> 31
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for K. marxianus promoter region.

<400> 70

atcgtctgca gactacacca gggcgtagta t 31

<210> 71
<211> 41
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for K. marxianus terminator region.

<400> 71
cgtagtatct gggccctatc tggtattatc taagaacgat t 41

<210> 72
<211> 43
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for K. marxianus terminator region.

<400> 72
tatacgtact tagggcccgc tatgcccctc actttactaa tat 43

<210> 73
<211> 16
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for K. marxianus promoter region.

<400> 73
cgtagtatct gggccc 16

<210> 74
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for K. marxianus promoter region.

<400> 74
tatacgtact tagggccc 18

<210> 75
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for K. marxianus promoter region.

<400> 75
caggacttgt tgcgtggcgc 20

<210> 76
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for K. marxianus promoter region.

<400> 76
gtatagatta acagtgtgtt 20

<210> 77
<211> 25
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for K. marxianus promoter region.

<400> 77
ccacgagcct catcacacga aaagt 25

<210> 78
<211> 27
<212> DNA
<213> Artificial

<220>
<223> G418 gene amplification primer

<400> 78
gtcttcgatt tgacctactt ctaccag 27

<210> 79
<211> 24
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for G418 gene

<400> 79
cgtaatagcg aagaggcccg cacc 24

<210> 80
<211> 28
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for G418 gene

<400> 80
atccgttctc aatcactaca gtagctta 28

<210> 81
<211> 30
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for S. cerevisiae xylulokinase gene

<400> 81
taggatccat gttgtgttca gtaattcaga 30

<210> 82

<211> 30
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for S. cerevisiae xylulokinase gene

<400> 82
taggatcctt agatgagagt cttttccagt 30

<210> 83
<211> 1803
<212> DNA
<213> Saccharomyces cerevisiae

<400> 83
atgttgtgtt cagtaattca gagacagaca agagaggttt ccaacacaat gtctttagac 60

tcatactatc ttgggtttga tctttcgacc caacaactga aatgtctcgc cattaaccag 120

gacctaaaaa ttgtccattc agaaacagtg gaatttgaaa aggatcttcc gcattatcac 180

acaaagaagg gtgtctatat acacggcgac actatcgaat gtcccgtagc catgtggtta 240

gaggctctag atctggttct ctcgaaatat cgcgaggcta aatttccatt gaacaaagtt 300

atggccgtct cagggtcctg ccagcagcac gggtctgtct actggtcctc ccaagccgaa 360

tctctgttag agcaattgaa taagaaaccg gaaaaagatt tattgcacta cgtgagctct 420

gtagcatttg caaggcaaac cgcccccaat tggcaagacc acagtactgc aaagcaatgt 480

caagagtttg aagagtgcat aggtgggcct gaaaaaatgg ctcaattaac agggtccaga 540

gcccatttta gatttactgg tcctcaaatt ctgaaaattg cacaattaga accagaagct 600

tacgaaaaaa caaagaccat ttctttagtg tctaattttt tgacttctat cttagtgggc 660

catcttgttg aattagagga ggcagatgcc tgtggtatga acctttatga tatacgtgaa 720

agaaaattca gtgatgagct actacatcta attgatagtt cttctaagga taaaactatc 780

agacaaaaat taatgagagc acccatgaaa aatttgatag cgggtaccat ctgtaaatat 840

tttattgaga agtacggttt caatacaaac tgcaaggtct ctcccatgac tggggataat 900

ttagccacta tatgttcttt acccctgcgg aagaatgacg ttctcgtttc cctaggaaca 960

agtactacag ttcttctggt caccgataag tatcacccct ctccgaacta tcatcttttc 1020

attcatccaa ctctgccaaa ccattatatg ggtatgattt gttattgtaa tggttctttg 1080

gcaagggaga ggataagaga cgagttaaac aaagaacggg aaaataatta tgagaagact 1140

aacgattgga ctctttttaa tcaagctgtg ctagatgact cagaaagtag tgaaaatgaa 1200

ttaggtgtat attttcctct gggggagatc gttcctagcg taaaagccat aaacaaaagg 1260

gttatcttca atccaaaaac gggtatgatt gaaagagagg tggccaagtt caaagacaag 1320

aggcacgatg ccaaaaatat tgtagaatca caggctttaa gttgcagggt aagaatatct 1380

cccctgcttt cggattcaaa cgcaagctca caacagagac tgaacgaaga tacaatcgtg 1440

aagtttgatt acgatgaatc tccgctgcgg gactacctaa ataaaaggcc agaaaggact 1500

ttttttgtag gtggggcttc taaaaacgat gctattgtga agaagtttgc tcaagtcatt 1560

ggtgctacaa agggtaattt taggctagaa acaccaaact catgtgccct tggtggttgt 1620

tataaggcca tgtggtcatt gttatatgac tctaataaaa ttgcagttcc ttttgataaa 1680

tttctgaatg acaattttcc atggcatgta atggaaagca tatccgatgt ggataatgaa 1740

aattgggatc gctataattc caagattgtc cccttaagcg aactggaaaa gactctcatc 1800

taa 1803

<210> 84
<211> 600
<212> PRT
<213> Saccharomyces cerevisiae

<400> 84

Met Leu Cys Ser Val Ile Gln Arg Gln Thr Arg Glu Val Ser Asn Thr
1 5 10 15

Met Ser Leu Asp Ser Tyr Tyr Leu Gly Phe Asp Leu Ser Thr Gln Gln
20 25 30

Leu Lys Cys Leu Ala Ile Asn Gln Asp Leu Lys Ile Val His Ser Glu
35 40 45

Thr Val Glu Phe Glu Lys Asp Leu Pro His Tyr His Thr Lys Lys Gly
50 55 60

Val Tyr Ile His Gly Asp Thr Ile Glu Cys Pro Val Ala Met Trp Leu
65 70 75 80

Glu Ala Leu Asp Leu Val Leu Ser Lys Tyr Arg Glu Ala Lys Phe Pro
85 90 95

Leu Asn Lys Val Met Ala Val Ser Gly Ser Cys Gln Gln His Gly Ser
100 105 110

Val Tyr Trp Ser Ser Gln Ala Glu Ser Leu Leu Glu Gln Leu Asn Lys
115 120 125

Lys Pro Glu Lys Asp Leu Leu His Tyr Val Ser Ser Val Ala Phe Ala
130 135 140

Arg Gln Thr Ala Pro Asn Trp Gln Asp His Ser Thr Ala Lys Gln Cys
145 150 155 160

Gln Glu Phe Glu Glu Cys Ile Gly Gly Pro Glu Lys Met Ala Gln Leu
165 170 175

Thr Gly Ser Arg Ala His Phe Arg Phe Thr Gly Pro Gln Ile Leu Lys
180 185 190

Ile Ala Gln Leu Glu Pro Glu Ala Tyr Glu Lys Thr Lys Thr Ile Ser

195 200 205

Leu Val Ser Asn Phe Leu Thr Ser Ile Leu Val Gly His Leu Val Glu
210 215 220

Leu Glu Glu Ala Asp Ala Cys Gly Met Asn Leu Tyr Asp Ile Arg Glu
225 230 235 240

Arg Lys Phe Ser Asp Glu Leu Leu His Leu Ile Asp Ser Ser Ser Lys
245 250 255

Asp Lys Thr Ile Arg Gln Lys Leu Met Arg Ala Pro Met Lys Asn Leu
260 265 270

Ile Ala Gly Thr Ile Cys Lys Tyr Phe Ile Glu Lys Tyr Gly Phe Asn
275 280 285

Thr Asn Cys Lys Val Ser Pro Met Thr Gly Asp Asn Leu Ala Thr Ile
290 295 300

Cys Ser Leu Pro Leu Arg Lys Asn Asp Val Leu Val Ser Leu Gly Thr
305 310 315 320

Ser Thr Thr Val Leu Leu Val Thr Asp Lys Tyr His Pro Ser Pro Asn
325 330 335

Tyr His Leu Phe Ile His Pro Thr Leu Pro Asn His Tyr Met Gly Met
340 345 350

Ile Cys Tyr Cys Asn Gly Ser Leu Ala Arg Glu Arg Ile Arg Asp Glu
355 360 365

Leu Asn Lys Glu Arg Glu Asn Asn Tyr Glu Lys Thr Asn Asp Trp Thr
370 375 380

Leu Phe Asn Gln Ala Val Leu Asp Asp Ser Glu Ser Ser Glu Asn Glu
385 390 395 400

Leu Gly Val Tyr Phe Pro Leu Gly Glu Ile Val Pro Ser Val Lys Ala
405 410 415

Ile Asn Lys Arg Val Ile Phe Asn Pro Lys Thr Gly Met Ile Glu Arg
420 425 430

Glu Val Ala Lys Phe Lys Asp Lys Arg His Asp Ala Lys Asn Ile Val
435 440 445

Glu Ser Gln Ala Leu Ser Cys Arg Val Arg Ile Ser Pro Leu Leu Ser
450 455 460

Asp Ser Asn Ala Ser Ser Gln Gln Arg Leu Asn Glu Asp Thr Ile Val

465 470 475 480

Lys Phe Asp Tyr Asp Glu Ser Pro Leu Arg Asp Tyr Leu Asn Lys Arg
485 490 495

Pro Glu Arg Thr Phe Phe Val Gly Gly Ala Ser Lys Asn Asp Ala Ile
500 505 510

Val Lys Lys Phe Ala Gln Val Ile Gly Ala Thr Lys Gly Asn Phe Arg
515 520 525

Leu Glu Thr Pro Asn Ser Cys Ala Leu Gly Gly Cys Tyr Lys Ala Met
530 535 540

Trp Ser Leu Leu Tyr Asp Ser Asn Lys Ile Ala Val Pro Phe Asp Lys
545 550 555 560

Phe Leu Asn Asp Asn Phe Pro Trp His Val Met Glu Ser Ile Ser Asp
565 570 575

Val Asp Asn Glu Asn Trp Asp Arg Tyr Asn Ser Lys Ile Val Pro Leu
580 585 590

Ser Glu Leu Glu Lys Thr Leu Ile
595 600

<210> 85
<211> 23
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for S. cerevisiae TEF1 promoter

<400> 85
gacactatag aatactcaag cta 23

<210> 86
<211> 29
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for S. cerevisiae TEF1 promoter

<400> 86
cgtctagatt cctcaccttg tcgtattat 29

<210> 87
<211> 42
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for S. cerevisiae TEF1 promoter

<400> 87

tattacgtat atgcgaattc cccacacacc atagcttcaa aa 42

<210> 88
<211> 45
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for S. cerevisiae TEF1 promoter

<400> 88
acgtattacg tagaattctg tcgtattata ctatgccgat atact 45

<210> 89
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for S. cerevisiae TEF1 promoter

<400> 89
tattacgtat atgcgaattc 20

<210> 90
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Amplification primer for S. cerevisiae TEF1 promoter

<400> 90
acgtattacg tagaattc 18

<210> 91
<211> 27
<212> DNA
<213> Artificial

<220>
<223> S. cerevisiae/HisG gene amplification primer

<400> 91
ggactccgcg catcgccgta ccacttc 27

<210> 92
<211> 20
<212> DNA
<213> Artificial

<220>
<223> S. cerevisiae/HisG gene amplification primer

<400> 92
tacgggacat tcgatagtgt 20

<210> 93
<211> 18
<212> DNA
<213> Artificial

<220>
<223> S. cerevisiae/HisG gene amplification primer

<400> 93
caggaaacag ctatgacc 18

<210> 94
<211> 24
<212> DNA
<213> Artificial

<220>
<223> S. cerevisiae/HisG gene amplification primer

<400> 94
gtagtgaaaa tgaattaggt gtat 24

<210> 95
<211> 18
<212> DNA
<213> Artificial

<220>
<223> S. cerevisiae xylulokkinase gene amplification primer

<400> 95
gttttcccag tcacgacg 18

<210> 96
<211> 40
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase gene amplification primer

<400> 96
accaaaaaca ctgcatagac taatattatt aatactatat 40

<210> 97
<211> 40
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase gene amplification primer

<400> 97
ggttgataat ttgtattttt gttattggta gcgctcgctc 40

<210> 98
<211> 40
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase terminator amplification primer

<400> 98
catgtttcaa aactgtgatt gaacgttatt tatgaatatg 40

<210> 99
<211> 40
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase terminator amplification primer

<400> 99
cctagggata ggttccgctc ctgttgggtt ataacgactc 40

<210> 100
<211> 40
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase terminator amplification primer

<400> 100
atctatgcat gccatgtttc aaaactgtga ttgaacgtta 40

<210> 101
<211> 40
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase terminator amplification primer

<400> 101
agttaagcat gccctaggga taggttccgc tcctgttggg 40

<210> 102
<211> 20
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase terminator amplification primer

<400> 102
atctatgcat gccatgtttc 20

<210> 103
<211> 20
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase terminator amplification primer

<400> 103
agttaagcat gccctaggga 20

<210> 104
<211> 25
<212> DNA

<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase gene amplification primer

<400> 104
atgaccaaca ctcaaaaagc cgttg 25

<210> 105
<211> 25
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase gene amplification primer

<400> 105
tcattctgga ccatcaatga tagtc 25

<210> 106
<211> 25
<212> DNA
<213> Artificial

<220>
<223> Hygromycin gene amplification primer

<400> 106
atgttcacaa gatgaaatat taccc 25

<210> 107
<211> 25
<212> DNA
<213> Artificial

<220>
<223> Hygromycin gene amplification primer

<400> 107
tgagcttgtt atcgatagcg gaaga 25

<210> 108
<211> 25
<212> DNA
<213> Artificial

<220>
<223> Hygromycin gene amplification primer

<400> 108
gtaggaacct ttggtgccac gtaag 25

<210> 109
<211> 21
<212> DNA
<213> Artificial

<220>
<223> Hygromycin gene amplification primer

<400> 109
ggccgcacta gagtcgacct g 21

1182A WO.ST25

<210> 110
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Piromyces xylose isomerase gene amplification primer

<400> 110
tgaacggtgc ctccactaac 20

<210> 111
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Piromyces xylose isomerase gene amplification primer

<400> 111
gccctctagg atcagcgggt 20

<210> 112
<211> 42
<212> DNA
<213> Artificial

<220>
<223> Piromyces xylose isomerase gene amplification primer

<400> 112
atcgtattcc tgcaggatgg ctaaggaata tttcccacaa at 42

<210> 113
<211> 44
<212> DNA
<213> Artificial

<220>
<223> Piromyces xylose isomerase gene amplification primer

<400> 113
atatcgaacc tgcaggttat tggtacatgg caacaatagc ttcg 44

<210> 114
<211> 33
<212> DNA
<213> Artificial

<220>
<223> L. helveticus lactate dehydrogenase gene amplification primer

<400> 114
tattagcatg cgacgtcggt aatctccgaa cag 33

<210> 115
<211> 23
<212> DNA
<213> Artificial

<220>
<223> L. helveticus lactate dehydrogenase gene amplification primer

<400> 115
gaaatgcatg cccacaggac ggg 23

<210> 116
<211> 37
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase gene amplification primer

<400> 116
attaatccgc gggggaaata cggacgggat tgaacgc 37

<210> 117
<211> 37
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase gene amplification primer

<400> 117
tattaaccgc ggcttattgt ggatcgaatt gtaatgt 37

<210> 118
<211> 20
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase gene amplification primer

<400> 118
tagcagcacg ttccttatat 20

<210> 119
<211> 20
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase gene amplification primer

<400> 119
ctatggtata gcgctgccta 20

<210> 120
<211> 28
<212> DNA
<213> Artificial

<220>
<223> Ura3 gene amplification primer

<400> 120
cttcaacaac aacaccactt gattcatg 28

<210> 121
<211> 20
<212> DNA

<213> Artificial

<220>
<223> Ura3 gene amplification primer

<400> 121
gtgcagttgg gttaagaata 20

<210> 122
<211> 26
<212> DNA
<213> Artificial

<220>
<223> S. cerevisiae xylulokinase gene amplification primer

<400> 122
gaggcagatg cctgtggtat gaacct 26

<210> 123
<211> 24
<212> DNA
<213> Artificial

<220>
<223> S. cerevisiae xylulokinase gene amplification primer

<400> 123
cgaaataaat ccgcggctcg tgct 24

<210> 124
<211> 36
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylose reductase gene amplification primer

<400> 124
attataccgc gggtagagag cacaaagtaa cgcaac 36

<210> 125
<211> 36
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylose reductase gene amplification primer

<400> 125
taatatccgc ggggctgtct tttgacaatt aggtcg 36

<210> 126
<211> 20
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylose reductase gene amplification primer

<400> 126
tcccctatat agatgatggc 20

<210> 127
<211> 26
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylose reductase gene amplification primer

<400> 127
ggtatctcaa acttttcggg tgcatt 26

<210> 128
<211> 26
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylose reductase gene amplification primer

<400> 128
tcccatgggt cgttaaatct caatcc 26

<210> 129
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Ura3 gene amplification primer

<400> 129
gcccactatc ctttgtcgag 20

<210> 130
<211> 37
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylose reductase gene amplification primer

<400> 130
attatagagc tcggtagaga gcacaaagta acgcaac 37

<210> 131
<211> 38
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylose reductase gene amplification primer

<400> 131
tattaagcgg ccgcggctgt cttttgacaa ttaggtcg 38

<210> 132
<211> 20
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylose reductase gene amplification primer

<400> 132
aggacgtgcg cacccacctg 20

<210> 133
<211> 20
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylose reductase gene amplification primer

<400> 133
gtttccacca cccagacaac 20

<210> 134
<211> 23
<212> DNA
<213> Artificial

<220>
<223> K. marxianus xylitol dehydrogenase gene amplification primer

<400> 134
caatgcaaag gtggtttatg taa 23

<210> 135
<211> 45
<212> DNA
<213> Artificial

<220>
<223> C. sonorensis xylitol dehydrogenase gene 5' flank amplification primer

<400> 135
actgtcgagc tcgtttaaac acctattcgg gagtcaatca accat 45

<210> 136
<211> 40
<212> DNA
<213> Artificial

<220>
<223> C. sonorensis xylitol dehydrogenase gene 5' flank amplification primer

<400> 136
actgacgcgt cgacgtatgt ataataaggt atgattctgg 40

<210> 137
<211> 39
<212> DNA
<213> Artificial

<220>
<223> C. sonorensis xylitol dehydrogenase gene 3' flank amplification primer

<400> 137
ggcccgcggc cgctaggcta gttttctaaa attttggtg 39

<210> 138
<211> 37
<212> DNA
<213> Artificial

<220>
<223> C. sonorensis xylitol dehydrogenase gene 3' flank amplification primer

<400> 138
gggacgggcc caagtatgag aaatattgat gatatag 37

<210> 139
<211> 26
<212> DNA
<213> Artificial

<220>
<223> C. sonorensis xylose reductase amplification primer

<220>
<221> misc_feature
<223> D = A/G/T; R = A/G; Y = C/T; W = A/T

<400> 139
gadgaraart ayccwccagg wttcta 26

<210> 140
<211> 24
<212> DNA
<213> Artificial

<220>
<223> C. sonorensis xylose reductase amplification primer

<220>
<221> misc_feature
<223> D = A/G/T; K = G/T; Y = C/T; W = A/T; R = A/G

<400> 140
ccadkyccaw ggrtyrttra atct 24

<210> 141
<211> 45
<212> DNA
<213> Artificial

<220>
<223> C. sonorensis xylose reductase 5' flank amplification primer

<400> 141
actgtcgagc tcgtttaaac cttcacctta aattccccaa ttgag 45

<210> 142
<211> 41
<212> DNA
<213> Artificial

<220>
<223> C. sonorensis xylose reductase 5' flank amplification primer

<400> 142

actgacgcgt cgactcttgt ttgattgtgt gttgattgat c 41

<210> 143
<211> 40
<212> DNA
<213> Artificial

<220>
<223> C. sonorensis xylose reductase 3' flank amplification primer

<400> 143
ggcccgcggc cgctaagcag ctagtatagg caagatgtag 40

<210> 144
<211> 36
<212> DNA
<213> Artificial

<220>
<223> C. sonorensis xylose reductase 3' flank amplification primer

<400> 144
gggacgggcc caactgtaat aatccgactt tcaacg 36

<210> 145
<211> 76
<212> DNA
<213> Artificial

<220>
<223> Piromyces xylose isomerase gene amplification primer

<400> 145
ggacatgcat gcatttgggg tacccaaggc cttccgctct agaaaacaat ggctaaggaa 60

tatttcccac aaattc 76

<210> 146
<211> 53
<212> DNA
<213> Artificial

<220>
<223> Piromyces xylose isomerase gene amplification primer

<400> 146
ccaatgcatt ggttcctgca gggaattcga caacatcaaa gtctgggtta gtg 53

<210> 147
<211> 53
<212> DNA
<213> Artificial

<220>
<223> S. cerevisiae xylulokinase gene amplification primer

<400> 147
aaggccttgc ggccgcctct agaaaacaat gttgtgttca gtaattcaga gac 53

<210> 148
<211> 35
<212> DNA

<213> Artificial

<220>
<223> S. cerevisiae xylulokinase gene amplification primer

<400> 148
gaaaaggcct tgttcaatgg aaatttagcc tcgcg 35

<210> 149
<211> 49
<212> DNA
<213> Artificial

<220>
<223> C aberensis xylose isomerase gene amplification primer

<220>
<221> misc_feature
<223> Y = C/T; M = A/C; R = A/G; S = G/C

<400> 149
aattaattcc tgcaggatgg ttaaggaata yttcycmrmc attsraaag 49

<210> 150
<211> 47
<212> DNA
<213> Artificial

<220>
<223> C aberensis xylose isomerase gene amplification primer

<400> 150
aattaattcc tgcaggttac atgtacatag caacaatagc ttcgtaa 47

<210> 151
<211> 1314
<212> DNA
<213> Cyllamyces aberensis

<400> 151
atggttaagg aatacttccc cgccattcaa aagattaagt tcgaaggtaa ggattccaag 60

aatccaatgg ccttccacta ttacgatgct gaaaaagaaa ttatgggtaa gaagatgaag 120

gattggttac gtttcgctat ggcctggtgg cacactcttt gtgccgaagg ttctgaccaa 180

ttcggtccag gtactaagac tttcccatgg aacgaaggta ccgacccaat tgaaaaggct 240

aaacaaaagg tcgatgctgg tttcgaaatc atgaccaagc ttggtattga acactactgt 300

ttccacgatg ttgatcttgt tgatgaaggt aagaatgttg aagaatacga aaagaacctt 360

aagactatcg ttgcttacct taaggaaaag caaaaggaaa ctggtattaa acttctctgg 420

agtactgcta acgtcttcgg tcacaaacgt tacatgaacg gtgcttccac taacccagac 480

tttgatgttg ttgcccgtgc tattgttcaa attaagaacg ctatggatgc cggtattgaa 540

ctcggtgccg aaaactacgt cttctggggt ggtcgtgaag gttacatgag tctccttaac 600

actgaccaaa agcgtgaaaa ggaacacatg gctatgatgc tcggtttagc cagagattac 660

gctcgttcca agggtttcaa gggtactttc ctcattgaac caaagccaat ggaaccaacc 720

aagcaccaat acgatgttga cactgaaact gtcattggtt tcctcagagc tcatggttta 780

gacaaggact tcaagattaa cattgaagtt aaccacgcta ctcttgctgg tcacactttc 840

gaacacgaac ttgcctgtgc tgttgatgcc ggtatgctcg gttctattga tgctaaccgt 900

ggtgattacc aaaacggttg ggatactgat caattcccaa ttgaccaata cgagcttgtt 960

caagcttgga tggaaattat ccgtggtggt ggtttcacta ctggtggtac taacttcgat 1020

gccaagactc gtcgtaactc taccgatctt gaagacatca ttattgccca catttctggt 1080

atggatgcta tggctcgtgc cctcgaaaac gctgccaagc tccttaccga atctccatac 1140

aagaagatga aggctgaccg ttacgcttcc ttcgactctg gtatgggtaa ggacttcgaa 1200

gatggtaagc ttaccttcga acaagtttac gaatacggta agaaggttaa cgaaccaaaa 1260

caaacctctg gtaaacaaga actttacgaa gctattgttg ctatgtacat gtaa 1314

<210> 152
<211> 437
<212> PRT
<213> Cyllamyces aberensis

<400> 152

Met Val Lys Glu Tyr Phe Pro Ala Ile Gln Lys Ile Lys Phe Glu Gly
1 5 10 15

Lys Asp Ser Lys Asn Pro Met Ala Phe His Tyr Tyr Asp Ala Glu Lys
20 25 30

Glu Ile Met Gly Lys Lys Met Lys Asp Trp Leu Arg Phe Ala Met Ala
35 40 45

Trp Trp His Thr Leu Cys Ala Glu Gly Ser Asp Gln Phe Gly Pro Gly
50 55 60

Thr Lys Thr Phe Pro Trp Asn Glu Gly Thr Asp Pro Ile Glu Lys Ala
65 70 75 80

Lys Gln Lys Val Asp Ala Gly Phe Glu Ile Met Thr Lys Leu Gly Ile
85 90 95

Glu His Tyr Cys Phe His Asp Val Asp Leu Val Asp Glu Gly Lys Asn
100 105 110

Val Glu Glu Tyr Glu Lys Asn Leu Lys Thr Ile Val Ala Tyr Leu Lys
115 120 125

Glu Lys Gln Lys Glu Thr Gly Ile Lys Leu Leu Trp Ser Thr Ala Asn
130 135 140

Val Phe Gly His Lys Arg Tyr Met Asn Gly Ala Ser Thr Asn Pro Asp
145 150 155 160

Phe Asp Val Val Ala Arg Ala Ile Val Gln Ile Lys Asn Ala Met Asp
165 170 175

Ala Gly Ile Glu Leu Gly Ala Glu Asn Tyr Val Phe Trp Gly Gly Arg
180 185 190

Glu Gly Tyr Met Ser Leu Leu Asn Thr Asp Gln Lys Arg Glu Lys Glu
195 200 205

His Met Ala Met Met Leu Gly Leu Ala Arg Asp Tyr Ala Arg Ser Lys
210 215 220

Gly Phe Lys Gly Thr Phe Leu Ile Glu Pro Lys Pro Met Glu Pro Thr
225 230 235 240

Lys His Gln Tyr Asp Val Asp Thr Glu Thr Val Ile Gly Phe Leu Arg
245 250 255

Ala His Gly Leu Asp Lys Asp Phe Lys Ile Asn Ile Glu Val Asn His
260 265 270

Ala Thr Leu Ala Gly His Thr Phe Glu His Glu Leu Ala Cys Ala Val
275 280 285

Asp Ala Gly Met Leu Gly Ser Ile Asp Ala Asn Arg Gly Asp Tyr Gln
290 295 300

Asn Gly Trp Asp Thr Asp Gln Phe Pro Ile Asp Gln Tyr Glu Leu Val
305 310 315 320

Gln Ala Trp Met Glu Ile Ile Arg Gly Gly Gly Phe Thr Thr Gly Gly
325 330 335

Thr Asn Phe Asp Ala Lys Thr Arg Arg Asn Ser Thr Asp Leu Glu Asp
340 345 350

Ile Ile Ile Ala His Ile Ser Gly Met Asp Ala Met Ala Arg Ala Leu
355 360 365

Glu Asn Ala Ala Lys Leu Leu Thr Glu Ser Pro Tyr Lys Lys Met Lys
370 375 380

Ala Asp Arg Tyr Ala Ser Phe Asp Ser Gly Met Gly Lys Asp Phe Glu
385 390 395 400

Asp Gly Lys Leu Thr Phe Glu Gln Val Tyr Glu Tyr Gly Lys Lys Val
405 410 415

Asn Glu Pro Lys Gln Thr Ser Gly Lys Gln Glu Leu Tyr Glu Ala Ile
420 425 430

Val Ala Met Tyr Met
435

<210> 153
<211> 39
<212> DNA
<213> Artificial

<220>
<223> K. marxianus Ura3 isolation primer

<400> 153
atatatgcat gccgtacctt agaatcctta tttgtatca 39

<210> 154
<211> 39
<212> DNA
<213> Artificial

<220>
<223> K. marxianus Ura3 isolation primer

<400> 154
atatatgcat gctaaactct ttttctttgg ttgtgaaat 39

<210> 155
<211> 1805
<212> DNA
<213> Kluyveromyces marxianus

<400> 155
ccgtacctta gaatccttat ttgtatcatc actcccagtc aacagtactc taatataatg 60
cgtatagtca aatctggccg gtcgaaacag ttttaatgga gttctcatat agaatgaagt 120
catctgataa accatagatc ttccaccagc agttaaagca ccaacaagtg acgaattctg 180
attggaaaga ccattctgct ttacttttag agcatcttgg tcttctgagc tcattatacc 240
tcaatcaaaa ctgaaattag gtgcctgtca cggctctttt tttactgtac ctgtgacttc 300
ctttcttatt tccaaggatg ctcatcacaa tacgcttcta gatctattat gcattataat 360
taatagttgt agctacaaaa ggtaaaagaa agtccggggc aggcaacaat agaaatcggc 420
aaaaaaaact acagaaatac taagagcttc ttccccattc agtcatcgca tttcgaaaca 480
agaggggaat ggctctggct agggaactaa ccaccatcgc ctgactctat gcactaacca 540
cgtgactaca tatatgtgat cgtttttaac atttttcaaa ggctgtgtgt ctggctgttt 600
ccattaattt tcactgatta agcagtcata ttgaatctga gctcatcacc aacaagaaat 660
actaccgtaa aagtgtaaaa gttcgtttaa atcatttgta aactggaaca gcaagaggaa 720
gtatcatcag ctagcccata aactaatcaa aggaggatgt cgactaagag ttactcggaa 780
agagcagctg ctcatagaag tccagttgct gccaagcttt taaacttgat ggaagagaag 840
aagtcaaact tatgtgcttc tcttgatgtt cgtaaaacaa cagagttgtt aagattagtt 900
gaggttttgg gtccatatat ctgtctattg aagacacatg tagatatctt ggaggatttc 960
agctttgaga ataccattgt gccgttgaag caattagcag agaaacacaa gtttttgata 1020

tttgaagaca ggaagtttgc cgacattggg aacactgtta aattacaata cacgtctggt 1080

gtataccgta tcgccgaatg gtctgatatc accaatgcac acggtgtgac tggtgcgggc 1140

attgttgctg gtttgaagca aggtgccgag gaagttacga aagaacctag agggttgtta 1200

atgcttgccg agttatcgtc caaggggtct ctagcgcacg gtgaatacac tcgtgggacc 1260

gtggaaattg ccaagagtga taaggacttt gttattggat ttattgctca aaacgatatg 1320

ggtggaagag aagagggcta cgattggttg atcatgacgc caggtgttgg tcttgatgac 1380

aaaggtgatg ctttgggaca acaatacaga actgtggatg aagttgttgc cggtggatca 1440

gacatcatta ttgttggtag aggtcttttc gcaaagggaa gagatcctgt agtggaaggt 1500

gagagataca gaaaggcggg atgggacgct tacttgaaga gagtaggcag atccgcttaa 1560

gagttctccg agaacaagca gaggttcgag tgtactcgga tcagaagtta caagttgatc 1620

gtttatatat aaactataca gagatgttag agtgtaatgg cattgcgcac attgtatacg 1680

ctacaagttt agtcacgtgc tagaagctgt ttttttgcac cgaaaatttt ttttttttt 1740

ttttttgttt tttggtgaag tacattatgt gaaatttcac aaccaaagaa aaagagttta 1800

gcatg 1805

<210> 156
<211> 22
<212> DNA
<213> Artificial

<220>
<223> K. marxianus Ura3 gene screening primer

<400> 156
agtgtattca ccgtgcgcta ga 22

<210> 157
<211> 22
<212> DNA
<213> Artificial

<220>
<223> K. marxianus Ura3 gene screening primer

<400> 157
cccattcagt catcgcattt cg 22

<210> 158
<211> 22
<212> DNA
<213> Artificial

<220>
<223> C. aberensis xylose isomerase gene internal screening primer

<400> 158
cgatgttgac actgaaactg tc 22

<210> 159
<211> 22
<212> DNA

<213> Artificial

<220>
<223> C. aberensis xylose isomerase gene internal screening primer

<400> 159
gaagtcctta cccataccag ag 22

<210> 160
<211> 42
<212> DNA
<213> Artificial

<220>
<223> B. thetaiotamicron xylose isomerase gene isolation primer

<400> 160
aattaattcc tgcaggatgg caacaaaaga atttttttccg gg 42

<210> 161
<211> 43
<212> DNA
<213> Artificial

<220>
<223> B. thetaiotamicron xylose isomerase gene isolation primer

<400> 161
aattaattcc tgcaggttag caatacatat tcagaattgc ctc 43

<210> 162
<211> 1317
<212> DNA
<213> Bacteroides thetaiotaomicron

<400> 162
atggcaacaa aagaattttt tccgggaatt gaaaagatta aatttgaagg taaagatagt 60

aagaacccga tggcattccg ttattacgat gcagagaagg tgattaatgg taaaaagatg 120

aaggattggc tgagattcgc tatggcatgg tggcacacat tgtgcgctga aggtggtgat 180

cagttcggtg gcggaacaaa gcaattccca tggaatggta atgcagatgc tatacaggca 240

gcaaaagata agatggatgc aggatttgaa ttcatgcaga agatgggtat cgaatactat 300

tgcttccatg acgtagactt ggtttcggaa ggtgccagtg tagaagaata cgaagctaac 360

ctgaaagaaa tcgtagctta tgcaaaacag aaacaggcag aaaccggtat caaactactg 420

tggggtactg ctaatgtatt cggtcacgcc cgctatatga acggtgcagc taccaatcct 480

gacttcgatg tagtagctcg tgctgctgtt cagatcaaaa atgcgattga tgcaacgatt 540

gaacttggcg gagagaatta tgtgttttgg ggtggtcgtg aaggctatat gtctcttctg 600

aacacagatc agaaacgtga aaaagaacac cttgcacaga tgttgacgat tgctcgtgac 660

tatgcccgtg cccgtggttt caaaggtact ttcctgatcg aaccgaaacc gatggaaccg 720

actaaacatc aatatgacgt agatacggaa actgtaatcg gcttcctgaa agctcatggt 780

ctggataagg atttcaaagt aaatatcgag gtgaatcacg caactttggc aggtcacact 840

ttcgagcatg aattggctgt agctgtagac aatggtatgt tgggctcaat tgacgccaat 900

cgtggtgact atcagaatgg ctgggataca gaccaattcc cgatcgacaa ttatgaactg 960

actcaggcta tgatgcagat tatccgtaat ggtggtctcg gtaccggtgg tacgaacttt 1020

gatgctaaaa cccgtcgtaa ttctactgat ctggaagata tctttattgc tcacatcgca 1080

ggtatggacg ctatggcccg tgcactcgaa agtgcagcgg ctctgctcga cgaatctccc 1140

tataagaaga tgctggctga ccgttatgct tcatttgatg ggggcaaagg taaagaattt 1200

gaagacggca agctgactct ggaggatgtg gttgcttatg caaaaacaaa aggcgaaccg 1260

aaacagacta gcggcaagca agaactttat gaggcaattc tgaatatgta ttgctaa 1317

<210> 163
<211> 438
<212> PRT
<213> Bacteroides thetaiotaomicron

<400> 163

Met Ala Thr Lys Glu Phe Phe Pro Gly Ile Glu Lys Ile Lys Phe Glu
1 5 10 15

Gly Lys Asp Ser Lys Asn Pro Met Ala Phe Arg Tyr Tyr Asp Ala Glu
20 25 30

Lys Val Ile Asn Gly Lys Lys Met Lys Asp Trp Leu Arg Phe Ala Met
35 40 45

Ala Trp Trp His Thr Leu Cys Ala Glu Gly Gly Asp Gln Phe Gly Gly
50 55 60

Gly Thr Lys Gln Phe Pro Trp Asn Gly Asn Ala Asp Ala Ile Gln Ala
65 70 75 80

Ala Lys Asp Lys Met Asp Ala Gly Phe Glu Phe Met Gln Lys Met Gly
85 90 95

Ile Glu Tyr Tyr Cys Phe His Asp Val Asp Leu Val Ser Glu Gly Ala
100 105 110

Ser Val Glu Glu Tyr Glu Ala Asn Leu Lys Glu Ile Val Ala Tyr Ala
115 120 125

Lys Gln Lys Gln Ala Glu Thr Gly Ile Lys Leu Leu Trp Gly Thr Ala
130 135 140

Asn Val Phe Gly His Ala Arg Tyr Met Asn Gly Ala Ala Thr Asn Pro
145 150 155 160

Asp Phe Asp Val Val Ala Arg Ala Ala Val Gln Ile Lys Asn Ala Ile
165 170 175

Asp Ala Thr Ile Glu Leu Gly Gly Glu Asn Tyr Val Phe Trp Gly Gly

180 185 190

Arg Glu Gly Tyr Met Ser Leu Leu Asn Thr Asp Gln Lys Arg Glu Lys
195 200 205

Glu His Leu Ala Gln Met Leu Thr Ile Ala Arg Asp Tyr Ala Arg Ala
210 215 220

Arg Gly Phe Lys Gly Thr Phe Leu Ile Glu Pro Lys Pro Met Glu Pro
225 230 235 240

Thr Lys His Gln Tyr Asp Val Asp Thr Glu Thr Val Ile Gly Phe Leu
245 250 255

Lys Ala His Gly Leu Asp Lys Asp Phe Lys Val Asn Ile Glu Val Asn
260 265 270

His Ala Thr Leu Ala Gly His Thr Phe Glu His Glu Leu Ala Val Ala
275 280 285

Val Asp Asn Gly Met Leu Gly Ser Ile Asp Ala Asn Arg Gly Asp Tyr
290 295 300

Gln Asn Gly Trp Asp Thr Asp Gln Phe Pro Ile Asp Asn Tyr Glu Leu
305 310 315 320

Thr Gln Ala Met Met Gln Ile Ile Arg Asn Gly Gly Leu Gly Thr Gly
325 330 335

Gly Thr Asn Phe Asp Ala Lys Thr Arg Arg Asn Ser Thr Asp Leu Glu
340 345 350

Asp Ile Phe Ile Ala His Ile Ala Gly Met Asp Ala Met Ala Arg Ala
355 360 365

Leu Glu Ser Ala Ala Ala Leu Leu Asp Glu Ser Pro Tyr Lys Lys Met
370 375 380

Leu Ala Asp Arg Tyr Ala Ser Phe Asp Gly Gly Lys Gly Lys Glu Phe
385 390 395 400

Glu Asp Gly Lys Leu Thr Leu Glu Asp Val Val Ala Tyr Ala Lys Thr
405 410 415

Lys Gly Glu Pro Lys Gln Thr Ser Gly Lys Gln Glu Leu Tyr Glu Ala
420 425 430

Ile Leu Asn Met Tyr Cys
435

<210> 164

<211> 19
<212> DNA
<213> Artificial

<220>
<223> B. thetaiotamicron xylose isomerase gene screening primer

<400> 164
ggcgtgaatg taagcgtga 19

<210> 165
<211> 21
<212> DNA
<213> Artificial

<220>
<223> B. thetaiotamicron xylose isomerase gene screening primer

<400> 165
ctgtagacaa tggtatgttg g 21

<210> 166
<211> 32
<212> DNA
<213> Artificial

<220>
<223> K. marxianus PDC promoter, Piromyces xylose isomerase and S. cerevisiae CYC1 terminator amplification primer

<400> 166
attgttaatt aactctctaa acttgaacag cc 32

<210> 167
<211> 34
<212> DNA
<213> Artificial

<220>
<223> K. marxianus PDC promoter, Piromyces xylose isomerase and S. cerevisiae CYC1 terminator amplification primer

<400> 167
attaatacgc gtagcttgca aattaaagcc ttcg 34