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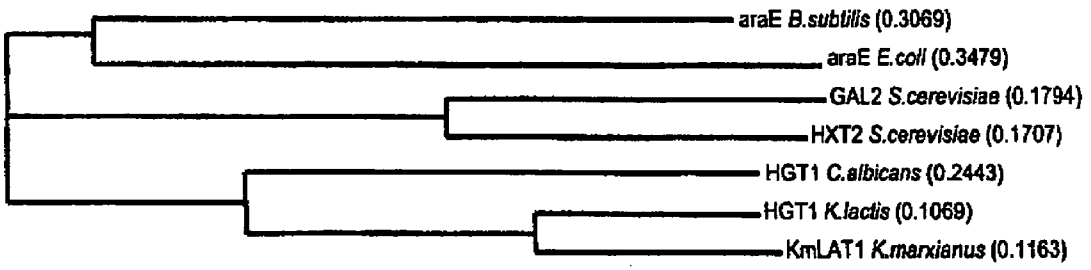
(54) Title: AN L-ARABINOSE FERMENTING YEAST



(57) Abstract: An L-arabinose utilizing yeast strain is provided for the production of ethanol by introducing and expressing bacterial *araA*, *araB* and araD genes. L-arabinose transporters are also introduced into the yeast to enhance the uptake of arabinose. The yeast carries additional genomic mutations enabling it to consume L-arabinose, even as the only carbon source, and to produce ethanol. Methods of producing ethanol include utilizing these modified yeast strains.

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AN L-ARABINOSE FERMENTING YEAST

CONTRACTUAL ORIGIN

[0001] The United States Government has rights in this invention under Contract No. DE-AC36-99GO10337 between the United States Department of Energy and the National Renewable Energy Laboratory, a Division of the Midwest Research Institute.

BACKGROUND

[0002] Fuel ethanol is a suitable alternative to fossil fuels. Ethanol may be produced from plant biomass, which is an economical and renewable resource that is available in large amounts. Examples of biomass include agricultural feedstocks, paper wastes, wood chips and so on. The sources of biomass vary from region to region based on the abundance of natural or agricultural biomass that is available in a particular region. For example, while sugar cane is the primary source of biomass used to produce ethanol in Brazil, corn-derived biomass, corn starch is a large source of biomass to produce ethanol in the United States. Other agricultural feedstocks include, by way of example: straw; grasses such as switchgrass; grains; and any other lignocellulosic or starch-bearing material.

[0003] A typical biomass substrate contains from 35-45% cellulose, 25-40% hemicellulose, and 15-30% lignin, although sources may be found that deviate from these general ranges. As is known in the art, cellulose is a polymer of glucose subunits, and hemicellulose contains mostly xylose. Arabinose is also a significant fermentable substrate that is found in biomass, such as corn fiber and many herbaceous crops in varying amounts. Other researchers have investigated the utilization of arabinose and hemicellulose, as reported by Hespell, R. B. 1998. *Extraction and characterization of hemicellulose from the corn fiber produced by corn wet-millingprocesses. J.* Agric. Food Chem. 46:2615-2619, and McMillan, J. D., and B. L. Boynton. 1994. *Arabinose utilization by xylose-fermentingyeasts and fungi.* Appl. Biochem. Biotechnol. 45-46:569-584. The two most abundant types of pentose that exist naturally are D-xylose and L-arabinose.

[0004] It is problematic that most of the naturally available ethanol-producing microorganisms are only capable of utilizing hexose sugar, such as glucose. This is confirmed by a review of the art, such as is reported by Barnett, J. A. 1976. The utilization of

sugars by yeasts. Adv. Carbohydr. Chem. Biochem. 32:125-234. Many types of yeast, especially Saccharomyces cerevisiae and related species, are very effective in fermenting glucose-based feedstocks into ethanol through anaerobic fermentation. However, these glucose-fermenting yeasts are unable to ferment xylose or L-arabinose, and are unable to grow solely on these pentose sugars. Although other yeast species, such as Pichia stipitis and Candida shehatae, can ferment xylose to ethanol, they are not as effective as Saccharomyces for fermentation of glucose and have a relatively low level of ethanol tolerance. Thus, the present range of available yeast are not entirely suitable for large scale industrial production of ethanol from biomass.

[0005] Most bacteria, including *E.* coli and Bacillus subtilis, utilize L-arabinose for aerobic growth, but they do not ferment L-arabinose to ethanol. These and other microorganisms, such as Zymononas mobilis, have also been genetically modified to produce ethanol from hexose or pentose. This has been reported, for example, in Deanda, K., M. Zhang, C. Eddy, and S. Picataggio. 1996, Development of an arabinose-fermenting Zymomonas mobilis strain by metabolic pathway engineering. Appl. Environ. Microbiol. 62:4465-4470; and Zhang, M., C. Eddy, K. Deanda, M. Finkelstein, and S. Picataggio. 1995 Metabolic engineering of a pentose metabolism pathway in ethanologenic Zymomonas mobilis. Science 267:240-243. However, it remains the case that the low alcohol tolerance of these non-yeast microorganisms limits their utility in the ethanol industry.

[0006] Much effort has been made over the last decade or so, without truly overcoming the problem of developing new strains that ferment xylose to generate ethanol. Such efforts are reported, for example, in Kotter, P., R. Amore, C. P. Hollenberg, and M. Ciriacy. 1990. Isolation and characterization of the Pichia stipitis xylitol dehydrogenase gene, XYL2, and construction of a xylose-utilizing Saccharomyces cerevisiae transformant. Curr. Genet. 18:493-500; and Wahlbom, C. F., and B. Hahn-Hagerdal. 2002 Recent studies have been conducted on yeast strains that potentially ferment arabinose. Sedlak, M., and N. W. Ho. 2001. Expression of *E.* coli araBAD operon encoding enzymes for metabolizing L-arabinose in Saccharomyces cerevisiae, Enzyme Microb. Technol. 28:16-24 discloses the expression of an E. coli araBAD operon encoding enzymes for metabolizing L-arabinose in Saccharomyces cerevisiae. Although this strain expresses araA, araB and araD proteins, it is incapable of producing ethanol.

[0007] United States Patent Application 10/983,951 by Boles and Becker discloses the creation of a yeast strain that may ferment L-arabinose. However, the overall yield is relatively low, at about 60% of theoretical value. The rate of arabinose transport into *S. cerevisiae* may be a limiting factor for complete utilization of the pentose substrate. Boles and Becker attempted to enhance arabinose uptake by overexpressing the GAL2-encoded galactose permease in *S. cerevisiae.* However, the rate of arabinose transport using galactose permease was still much lower when compared to that exhibited by non-conventional yeast such as *Kluyveromyces marxianus.* Another limitation that may have contributed to the low yield of ethanol in the modified strain of Becker and Boles is the poor activity of the L-arabinose isomerase encoded by the bacterial araA gene. Although Becker and Boles used an araA gene from B. *subtilis* instead of one from E. *coli,* the specific activity of the enzyme was still low. Other workers in the field have reported that low isomerase activity is a bottleneck in L-arabinose utilization by yeast.

[0008] There remains a need for new arabinose-fermenting strains that are capable of producing ethanol at high yield. There is further a need to identify novel arabinose transporters for introduction into *Saccharomyces cerevisiae* to boost the production of ethanol from arabinose.

[0009] The foregoing examples of the related art and limitations related therewith are intended to be illustrative and not exclusive. Other limitations of the related art will become apparent to those of skill in the art upon a reading of the specification and a study of the drawings.

SUMMARY

[0010] The following embodiments and aspects thereof are described and illustrated in conjunction with systems, tools and methods which are meant to be exemplary and illustrative, not limiting in scope. In various embodiments, one or more of the above-described problems have been reduced or eliminated, while other embodiments are directed to other improvements.

[0011] The presently disclosed instrumentalities overcome some of the problems outlined above and advance the art by providing new yeast strains that are capable of using L-arabinose to produce ethanol at a relatively high yield. Since the yeast galactose permease may faciliate uptake of arabinose, any Gal^+ strain possessing endogenous galactose permease activity may be used as described below. Although *S. cerevisiae* is used by way of example,

the scope of coverage extends to any organisms possessing endogenous pathways to generate ethanol from arabinose and to organisms into which components of such arabinose metabolic pathways or arabinose transporters may be introduced. The use of S. cerevisiae is preferred.

[0012] In a brief overview of the recombinant technique, the endogenous yeast aldose reductase (AR) gene is disrupted by replacing the AR coding sequence with the yeast LEU2 gene. Because the yeast aldose reductase is the first enzyme to metabolize arabinose in yeast, an AR^- strain is used to reduce diversion of arabinose to unwanted byproducts and to prevent possible inhibition of the isomerase by arabitol. The bacterial *araA, araB*, and *araD* genes are cloned into appropriate yeast expression vectors. The expression constructs containing all three ara genes are introduced in the AR^- strain and the transformants were capable of making ethanol from L-arabinose.

[0013] In another aspect of this disclosure, two novel arabinose transporter genes, termed *KmLAT1* and *PgLAT2*, have been cloned and characterized from two non-conventional yeast species, Kluyveromyces *marxianus* and Pichia guilliermondii (also known as Candida *guilliermondii*), respectively. Both Kluyveromyces *marxianus* and Pichia guilliermondii are efficient utilizers of L-arabinose, which renders them ideal sources for cloning L-arabinose transporter genes.

[0014] The *KmLAT1* gene may be isolated using functional complementation of an adapted *S.* cerevisiae strain that could not grow on L-arabinose because it lacked sufficient L-arabinose transport activity. KmLat1 protein has a predicted length of 556 amino acids encoded by a single ORF of 1668 bp. It is a transmembrane protein having high homology to sugar transporters of many different yeast species. When KmLat1 is expressed in S. cerevisiae, transport assays using labeled L-arabinose show that this transporter has the kinetic characteristics of a low affinity arabinose transporter, with K_m = 230 mM and V_{max} = 55 nmol/mg·min. Transport of L-arabinose by KmLat1 is not significantly inhibited by common uncoupling agents but is out-competed by glucose, galactose, xylose, and maltose.

[0015] The *PgLAT2* gene may be isolated using the technique of differential display from Pichia guilliermondii. The *PgLAT2* gene has an ORF of 1617 nucleotides encoding a protein with a predicted length of 539 amino acids. When *PgLAT2* is expressed in S. cerevisiae, transport assays show that this transporter has almost identical L-arabinose transport kinetics as that of wildtype Pichia guilliermondii. The PgLat2 transporter when expressed in S. cerevisiae has a K_m of 0.07 mM and V_{max} of 18 nmol/mg-min for L-arabinose

transport. Inhibition experiments show significant inhibition of the PgLat2 transporter by protonophores (e.g., NaN_3, DNP, and CCP) and H+-adenosine triphosphatase (ATPase) inhibitors (e.g., DESB and DCCD) similar to inhibition in wildtype P. *guilliermondii*. Competition experiments show that L-arabinose uptake by the PgLat2 transporter is inhibited by glucose, galactose, xylose and to a lesser extent by maltose.

[0016] The transport kinetics of *S.* cerevisiae Gal2p have been measured and compared to those of KmLat1. The *S.* cerevisiae GAL2 gene (SEQ ID NO 5) under control of a TDH3 promoter exhibits 28 times greater (8.9 nmol/mg·min) L-arabinose transport rate as compared to GAL2 gene under control of a ADH1 promoter. The GAL2-encoded permease (SEQ ID NO 6) shows a K_t of 550 m*M* and a V_{max} of 425 nmol/mg·min for L-arabinose transport and a K_m of 25 m*M* and a V_{max} of 76 nmol/mg·min for galactose transport. Although L-arabinose transport by both KmLATI and GAL2 encoded permeases is out-competed by glucose or galactose, the inhibitory effects of glucose or galactose are greater on the GAL2 encoded permease than on the KmLATI encoded transporter.

[0017] It is further disclosed here that a *S.* cerevisiae strain may be transformed with different combinations of the KmLATI and *PgLAT2* transporter genes and a plasmid carrying the GAL2 gene native to *S.* cerevisiae. The doubling time for the PgLat2p and Gal2p co-expressing cells grown on L-arabinose is markedly shorter than that of the cells expressing only Gal2p, suggesting that L-arabinose uptake may have been enhanced in these cells. In addition, the PgLat2p and Gal2p co-expressing cells appear to grow to a higher optical density at saturation, suggesting that this strain may be able to utilize the L-arabinose in the medium more completely. This conclusion is supported by HPLC analysis which shows significantly less residual L-arabinose in the culture of cells expressing PgLat2p and Gal2p.

[0018] In one embodiment, the transformed strains that carry the new transporter genes may be further transformed with plamids carrying three bacterial genes, *araA*, *araB* and *araC*, which encode proteins that may be utilized for arabinose utilization and fermentation. In another embodiment, the bacterial genes, *araA*, *araB* and *araC*, may be transformed into a yeast strain that does not carry any of the new transporter genes.

[0019] In addition to the exemplary aspects and embodiments described above, further aspects and embodiments will become apparent by reference to the drawings and by study of the following descriptions.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Exemplary embodiments are illustrated in referenced figures of the drawings. It is intended that the embodiments and figures disclosed herein are to be considered illustrative rather than limiting.

[0021] Figure 1 shows the relationship between *KmLAT1* and other transporters based on the neighbor-joining method (Saitou and Nei 1987).

[0022] Figure 2 shows the DNA (SEQ ID NO. 1) sequence of *Kluyveromyces marxianus* Km*LAT1*, and the predicted protein sequence (SEQ ID NO. 2).

[0023] Figure 3 is a schematic presentation of the arabinose metabolic pathway in recombinant yeast containing proteins encoded by three bacterial genes *araB*, *araA*, and *araD*.

[0024] Figure 4 shows the library insert from genomic *K. marxianus* DNA complements adapted *S. cerevisiae* for growth on L-arabinose. Cloning into the library expression vector is at the indicated *BamHI* restriction sites. The black block arrow is the L-arabinose transporter ORF responsible for complementation (Km*LAT1*). The block arrow with vertical stripes is the interrupted transporter ORF. The block arrow with the horizontal stripes is an un-related ORF ligated in place gratuitously during library construction. The *Sau3AI* restriction site where the transporter ORF was interrupted is shown. The primer used for PCR based genomic walking in *K. marxianus* is shown.

[0025] Figure 5 shows the growth curve of *S. cerevisiae* expressing *KmLAT1* (Δ), *GAL2* (■) or a control vector (♦) on 2% L-arabinose.

[0026] Figure 6. (A): Eadie-Hofstee plot of L-arabinose uptake by KmLat1 (♦) or Gal2 (■) expressed in *S. cerevisiae* grown on 2% L-arabinose. (B): Comparison of Eadie-Hofstee plots of KmLat1 expressed in *S. cerevisiae* (♦) and wild type transport activity of *K. marxianus* (Δ) both grown on 2% L-arabinose.

[0027] Figure 7 shows the DNA (SEQ ID NO. 3) sequence of *Pichia guilliermondii PgLAT2*, and the predicted protein sequence (SEQ ID NO. 4).

[0028] Figure 8 shows the induction of L-arabinose transport in *P. guilliermondii*. Uptake of 13 mM labeled sugar was assayed for cells grown in minimal media containing 2% L-arabinose, D-galactose or D-xylose. White bars indicate labeled L-arabinose transport.

Black bars indicate labeled galactose transport. Bars with vertical stripes indicate labeled xylose transport.

[0029] Figure 9 shows the sugar transport competition analysis in *P. guilliermondii* grown in minimal L-arabinose medium.

[0030] Figure 10 shows the transport kinetics of L-arabinose by the PgLAT2 transporter expressed in *S. cerevisiae*. Open triangles indicate transport for wild type *P. guilliermondii* grown on L-arabinose. Black diamonds indicate transport for PgLAT2 expressed in *S. cerevisiae* grown on L-arabinose.

[0031] Figure 11 shows comparison of the growth curves in 0.2% L-arabinose for *S. cerevisiae* cells expressing either Gal2p alone or both Gal2p and PgLat2. The maximum growth density and growth rate are significantly enhanced in the strain expressing both Gal2p and PgLat2.

[0032] Figure 12 shows the growth curves of BFY001 (parent) (black square) and BFY002 (ΔAR) (black triangle) on glucose and xylulose.

[0033] Figure 13 shows the diagrams of the expression plasmids with *araB*, *araA*, and *araD* genes carrying the *His3* selectable marker.

[0034] Figure 14 shows the diagrams of the expression plasmids with *araB*, *araA*, and *araD* genes carrying the *Ura3* selectable marker.

[0035] Figure 15 shows histogram with the result of ethanol production in whole-cell fermentation using yeast cells expressing the three bacterial genes *araB*, *araA*, and *araD*.

[0036] Figure 16 shows histogram with the result of ethanol production in cell-free fermentation using yeast cells expressing the three bacterial genes *araB*, *araA*, and *araD*.

[0037] Figure 17 plots ethanol production using yeast cells expressing the three bacterial genes *araB*, *araA*, and *araD*, as a function of the incubation time in a cell-free fermentation system.

[0038] Figure 18 shows histogram with the result of ethanol production in cell-free fermentation using yeast cells expressing the three bacterial genes *araB*, *araA*, and *araD*.

[0039] Figure 19 plots ethanol production using yeast cells expressing the three bacterial genes araB, araA, and araD, under single- and mixed-sugar fermentations.

DETAILED DESCRIPTION

[0040] There will now be shown and described methods for producing transgenic yeast that are capable of metabolizing arabinose and producing ethanol. In the discussion below, parenthetical mention is made to publications from the references section for a discussion of related procedures that may be found useful from a perspective of one skilled in the art. This is done to demonstrate what is disclosed by way of nonlimiting example.

[0041] The following definitions are provided to facilitate understanding of certain terms used frequently herein and are not meant to limit the scope of the present disclosure:

[0042] "Amino acid" refers to any of the twenty naturally occurring amino acids as well as any modified amino acid sequences. Modifications may include natural processes such as posttranslational processing, or may include chemical modifications which are known in the art. Modifications include but are not limited to: phosphorylation, ubiquitination, acetylation, amidation, glycosylation, covalent attachment of flavin, ADP-ribosylation, cross linking, iodination, methylation, and the like.

[0043] "Antibody" refers to a generally Y-shaped molecule having a pair of antigen binding sites, a hinge region and a constant region. Fragments of antibodies, for example an antigen binding fragment (Fab), chimeric antibodies, antibodies having a human constant region coupled to a murine antigen binding region, and fragments thereof, as well as other well known recombinant antibodies are included in this definition.

[0044] "Antisense" refers to polynucleotide sequences that are complementary to target "sense" polynucleotide sequence.

[0045] "Biomass" refers collectively to organic non-fossil material. "Biomass" in the present disclosure refers particularly to plant material that is used to generate fuel, such as ethanol. Examples of biomass includes but are not limited to corn fiber, dried distiller's grain, jatropha, manure, meat and bone meal, miscanthus, peat, plate waste, landscaping waste, maize, rice hulls, silage, stover, maiden grass, switchgrass, whey, and bagasse from sugarcane.

[0046] "Complementary" or "complementarity" refers to the ability of a polynucleotide in a polynucleotide molecule to form a base pair with another polynucleotide in a second polynucleotide molecule. For example, the sequence A-G-T is complementary to the sequence T-C-A. Complementarity may be partial, in which only some of the

polynucleotides match according to base pairing, or complete, where all the polynucleotides match according to base pairing.

[0047] The term "derivative" refers to compounds that are derived from a predecessor compound by way of chemical or physical modification. For example, a compound is a sugar derivatives if it is formed by oxidization of one or more terminal groups to carboxylic acids, by reduction of a carbonyl group, by substitution of hydrogen(s), amino group(s), thiol group(s), etc, for one or more hydroxyl groups on a sugar, or if it is formed by phosphorylation on a sugar molecule.

[0048] "Expression" refers to transcription and translation occurring within a host cell. The level of expression of a DNA molecule in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present within the cell or the amount of DNA molecule encoded protein produced by the host cell (Sambrook et al., 1989, *Molecular cloning: A Laboratory Manual*, 18.1-18.88).

[0049] "Fusion protein" refers to a first protein attached to a second, heterologous protein. Preferably, the heterologous protein is fused via recombinant DNA techniques, such that the first and second proteins are expressed in frame. The heterologous protein may confer a desired characteristic to the fusion protein, for example, a detection signal, enhanced stability or stabilization of the protein, facilitated oligomerization of the protein, or facilitated purification of the fusion protein. Examples of heterologous proteins useful as fusion proteins include molecules having full-length or partial protein sequence of KmLat1 or PgLat2. Further examples include peptide tags such as histidine tag (6-His), leucine zipper, substrate targeting moieties, signal peptides, and the like. Fusion proteins are also meant to encompass variants and derivatives of KmLat1 or PgLat2 polypeptides that are generated by conventional site-directed mutagenesis and more modern techniques such as directed evolution, discussed infra.

[0050] "Genetically engineered" refers to any recombinant DNA or RNA method used to create a prokaryotic or eukaryotic host cell that expresses a protein at elevated levels, at lowered levels, or in a mutated form. In other words, the host cell has been transfected, transformed, or transduced with a recombinant polynucleotide molecule, and thereby been altered so as to cause the cell to alter expression of the desired protein. Methods and vectors for genetically engineering host cells are well known in the art; for example various techniques are illustrated in Current Protocols in Molecular Biology, Ausubel et al., eds.

(Wiley & Sons, New York, 1988, and quarterly updates). Genetic engineering techniques include but are not limited to expression vectors, targeted homologous recombination and gene activation (see, for example, U.S. Patent No. 5,272,071 to Chappel) and trans activation by engineered transcription factors (see, for example, Segal et al., 1999, *Proc Natl Acad Sci USA* 96(6):2758-63). Genetic engineering also encompasses any mutagenesis techniques wherein a cell is exposed to chemicals to induce errors in DNA replication or to accelerate gene recombination. The term "spontaneous mutation" refers to mutations that occurs at a much lower rate as a result of genetic recombination or DNA replication errors that occur naturally from generation to generation.

[0051] "Heterologous" refers to DNA, RNA and/or polypeptides derived from different organisms or species, for example a bacterial polypeptide is heterologous to yeast.

[0052] "Homology" refers to a degree of similarity between polynucleotides, having significant effect on the efficiency and strength of hybridization between polynucleotide molecules. The term also refers to a degree of similarity between polypeptides. Two polypeptides having greater than or equal to about 60% similarity are presumptively homologous.

[0053] "Host," "Host cell" or "host cells" refers to cells expressing a heterologous polynucleotide molecule. The term "heterologous" means non-native. For instance, when a gene that is not normally expressed in an organism is introduced and expressed in that host organism, such an expression is heterologous. Host cells of the present disclosure express polynucleotides encoding KmLAT1 or PgLAT2 or a fragment thereof. Examples of suitable host cells useful in the present disclosure include, but are not limited to, prokaryotic and eukaryotic cells. Specific examples of such cells include bacteria of the genera *Escherichia, Bacillus,* and *Salmonella,* as well as members of the genera *Pseudomonas, Streptomyces,* and *Staphylococcus*; fungi, particularly filamentous fungi such as *Trichoderma* and *Aspergillus*, *Phanerochaete chrysosporium* and other white rot fungi; also other fungi including *Fusaria*, molds, and yeast including *Saccharomyces* sp., *Pichia* sp., and *Candida* sp. and the like; plants e.g. *Arabidopsis,* cotton, barley, tobacco, potato, and aquatic plants and the like; SF9 insect cells (Summers and Smith, 1987, *Texas Agriculture Experiment Station Bulletin*, 1555), and the like. Other specific examples include mammalian cells such as human embryonic kidney cells (293 cells), Chinese hamster ovary (CHO) cells (Puck et al., 1958, *Proc. Natl. Acad. Sci. USA* 60, 1275-1281), human cervical carcinoma cells (HELA) (ATCC CCL 2), human liver

cells (Hep G2) (ATCC HB8065), human breast cancer cells (MCF-7) (ATCC HTB22), human colon carcinoma cells (DLD-1) (ATCC CCL 221), Daudi cells (ATCC CRL-213), murine myeloma cells such as P3/NSI/1-Ag4-1 (ATCC TIB-18), P3X63Ag8 (ATCC TIB-9), SP2/0-Ag14 (ATCC CRL-1581) and the like. The most preferred host is *Saccharomyces cerevisiae*.

[0054] "Hybridization" refers to the pairing of complementary polynucleotides during an annealing period. The strength of hybridization between two polynucleotide molecules is impacted by the homology between the two molecules, stringency of the conditions involved, the melting temperature of the formed hybrid and the G:C ratio within the polynucleotides.

[0055] "Identity" refers to a comparison of two different DNA or protein sequences by comparing pairs of nucleic acid or amino acids within the two sequences. Methods for determining sequence identity are known. See, for example, computer programs commonly employed for this purpose, such as the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wisconsin), that uses the algorithm of Smith and Waterman, 1981, *Adv. Appl. Math., 2:* 482-489.

[0056] "Isolated" refers to a polynucleotide or polypeptide that has been separated from at least one contaminant (polynucleotide or polypeptide) with which it is normally associated. For example, an isolated polynucleotide or polypeptide is in a context or in a form that is different from that in which it is found in nature.

[0057] "Nucleic acid sequence" refers to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along a polypeptide chain. The deoxyribonucleotide sequence thus codes for the amino acid sequence.

[0058] "Polynucleotide" refers to a linear sequence of nucleotides. The nucleotides may be ribonucleotides, or deoxyribonucleotides, or a mixture of both. Examples of polynucleotides in this context include single and double stranded DNA, single and double stranded RNA, and hybrid molecules having mixtures of single and double stranded DNA and RNA. The polynucleotides may contain one or more modified nucleotides.

[0059] "Protein," "peptide," and "polypeptide" are used interchangeably to denote an amino acid polymer or a set of two or more interacting or bound amino acid polymers.

[0060] "Purify," or "purified" refers to a target protein makes up for at least about 90% of a composition. In other words, it refers to a target protein that is free from at least 5-10% of contaminating proteins. Purification of a protein from contaminating proteins may be accomplished using known techniques, including ammonium sulfate or ethanol precipitation, acid precipitation, heat precipitation, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, size-exclusion chromatography, and lectin chromatography. Various protein purification techniques are illustrated in *Current Protocols in Molecular Biology*, Ausubel et al., eds. (Wiley & Sons, New York, 1988, and quarterly updates).

[0061] "Selectable marker" refers to a marker that identifies a cell as having undergone a recombinant DNA or RNA event. Selectable markers include, for example, genes that encode antimetabolite resistance such as the DHFR protein that confers resistance to methotrexate (Wigler et al, 1980, *Proc Natl Acad Sci* USA 77:3567; O'Hare et al., 1981, *Proc Natl Acad Sci USA*, 78:1527), the GPT protein that confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *PNAS USA*, 78:2072), the neomycin resistance marker that confers resistance to the aminoglycoside G-418 (Calberre-Garapin et al., 1981, *J Mol Biol*, 150:1), the Hygro protein that confers resistance to hygromycin B (Santerre et al., 1984, *Gene* 30:147), and the Zeocin™ resistance marker (Invitrogen). In addition, the herpes simplex virus thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes may be employed in tk^-, $bgprt^-$ and $aprt^-$ cells, respectively.

[0062] "Transform" means the process of introducing a gene into a host cell. The gene may be foreign in origin, but the gene may also derive from the host. A transformed host cell is termed a "transformant." The introduced gene may be integrated onto the chromosome of the host, or the gene may remain on a stand-alone vector independent of the host chromosomes.

[0063] "Variant", as used herein, means a polynucleotide or polypeptide molecule that differs from a reference molecule. Variants may include nucleotide changes that result in amino acid substitutions, deletions, fusions, or truncations in the resulting variant polypeptide when compared to the reference polypeptide.

[0064] "Vector," "extra-chromosomal vector" or "expression vector" refers to a first polynucleotide molecule, usually double-stranded, which may have inserted into it a second polynucleotide molecule, for example a foreign or heterologous polynucleotide. The heterologous polynucleotide molecule may or may not be naturally found in the host cell, and may be, for example, one or more additional copy of the heterologous polynucleotide naturally present in the host genome. The vector is adapted for transporting the foreign polynucleotide molecule into a suitable host cell. Once in the host cell, the vector may be capable of integrating into the host cell chromosomes. The vector may optionally contain additional elements for selecting cells containing the integrated polynucleotide molecule as well as elements to promote transcription of mRNA from transfected DNA. Examples of vectors useful in the methods disclosed herein include, but are not limited to, plasmids, bacteriophages, cosmids, retroviruses, and artificial chromosomes.

[0065] For purpose of this disclosure, unless otherwise stated, the techniques used may be found in any of several well-known references, such as: *Molecular Cloning: A Laboratory Manual* (Sambrook et al. (1989) Molecular cloning: A Laboratory Manual), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991 Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, 3d., (1990) Academic Press, Inc.), *PCR Protocols: A Guide to Methods and Applications* (Innis et al. (1990) Academic Press, San Diego, CA), Culture of Animal Cells: A Manual of Basic Technique, 2nd ed. (R.I. Freshney (1987) Liss, Inc., New York, NY), *and Gene Transfer and Expression Protocols*, pp 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.).

[0066] Unless otherwise indicated, the term "yeast," "yeast strain" or "yeast cell" refers to baker's yeast, *Saccharomyces cerevisiae*. Other yeast species, such as *Kluyveromyces marxianus* or *Pichia guilliermondii*, are referred to as non-conventional yeast in this disclosure. Strains of *S. cerevisiae*, depository information, and plasmids used for this disclosure are listed in Table 1, 2 and Table 3, respectively. The yeast *Kluyveromyces marxianus* CBS-1089 is obtained from the Centraalbureau voor Schimmelcultures (CBS) collection. *Pichia guilliermondii* NRRL Y-2075 is obtained from the Agricultural Research Service Culture Collection (NRRL).

Table 1. *S. cerevisiae* Strains Used in this Disclosure

Strain	Genotype	Plasmids
BFY001	*MATa ura3-52 trp1-Δ63 his3-Δ200 leu2-Δ1*	
BFY002	*MATa ura3-52 trp1-Δ63 his3-Δ200 leu2-Δ1 yhr104w::LEU2*	
BFY507	*MATa ura3-52 trp1-Δ63 his3-Δ200 leu2-Δ1 yhr104w::LEU2* adapted for growth on L-arabinose	p138, p42
BFY518	same as BFY507	p138
BFY566	same as BFY518	p138, p171
BFY590	same as BFY518 *gal2Δ::HIS3*	p138
BFY597	same as BFY590	p138, p42
BFY598	same as BFY590	p138, p187
BFY012	same as BFY002	pBFY004, pBFY013, pBFY012
BFY013	same as BFY002	pBFY007, pBFY016, pBFY014
BFY014	same as BFY002	pBFY007, pBFY015, pBFY017
BFY015	same as BFY002	pBFY005, pBFY016, pBFY019
BFY016	same as BFY002	pBFY005, pBFY018, pBFY017
BFY017	same as BFY002	pBFY009, pBFY018, pBFY014
BFY018	same as BFY002	pBFY009, pBFY015, pBFY019
BFY057	*MATa his3D1 leu2D0 ura3D0 met15D0 gal80D::G418 yhr104w::LEU2*	
BFY534	same as BFY057	p144, p165
BFY535	same as BFY057	p144, pBFY13
BFY605	same as BFY590	p244
BFY625	*MATa his3Δ1 leu2Δ0 ura3Δ0 trp1Δ met15Δ0 gal80Δ::G418* adapted for growth on L-arabinose	pBFY12, pBFY13, p138
BFY626	Same as BFY625	pBFY12, p138, p204

[0067] Yeast strains may be grown on liquid or solid media with 2% agar for solid media. Where appropriate, some amino acids or nucleic acids are purposely left out from the media for plasmid maintenance. Growth conditions are typically 30°C unless otherwise indicated, with shaking in liquid cultures. Anaerobic conditions are generally more favorable to metabolize the various sugars to ethanol.

[0068] A number of the yeast strains listed in Table 1 have been deposited at the American Type Culture Collection (ATCC) in accordance with the provisions of the Budapest Treatey on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedures. In each instance, the yeast strain was deposited by the inventors listed herein on March 16, 2007 at American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110 U.S.A.

Table 2. Depository Information

Yeast Strain/Accession Number
BFY013/
BFY534/
BFY598/
BFY626/

Table 3. Plasmids Used in this Disclosure

Plasmid	Marker and expressed genes
p42	*URA3*, *GAL2* over-expression
p138	*TRP1*, *B. subtilis araA*, *E. coli araB*, *E. coli araD*
p144	*E. coli araB,D*; *B. subtilis araA* in pBFY012
p165	*HIS3*, *GAL2* over-expression
p171	*HIS3*, 8.8 kb *K. marxianus* genomic DNA fragment
p187	*URA3*, *KmLAT1* over-expression plasmid
p204	*HIS3*, *PgLAT2* over-expression plasmid
p244	*URA3*, *PgLAT2* over-expression plasmid
pBFY004	control 2μ vector with *PGK* promoter, *GAL10* terminator and Trp1 marker
pBFY005	*E. coli araB* in pBFY004
pBFY007	*E. coli araA* in pBFY004
pBFY009	*E. coli araD* in pBFY004
pBFY012	control 2μ vector with *PGK* promoter, *GAL10* terminator and Ura3 marker
pBFY013	control 2μ vector with *PGK* promoter, *GAL10* terminator and His3 marker
pBFY014	*E. coli araB* in pBFY012
pBFY015	*E. coli araB* in pBFY013
pBFY016	*E. coli araD* in pBFY013
pBFY017	*E. coli araD* in pBFY012
pBFY018	*E. coli araA* in pBFY013
pBFY019	*E. coli araA* in pBFY012

[0069] Yeast cells may be grown in rich media YPD or minimum media conventionally used in the field. YPD medium contains about 1% yeast extract, 2% peptone and 2% dextrose. Yeast minimum media typically contains 0.67% of yeast nitrogen base ("YNB") without amino acids supplemented with appropriate amino acids or purine or pyrimidine bases. An amount of sugar, typically 2% unless otherwise indicated, may be used as carbon source, including glucose (dextrose), galactose, maltose or L-arabinose among

others. Adaptation for growth on L-arabinose is performed as described in, for example, Becker and Boles (2003) with modifications as detailed in Example 3.

[0070] Over-expression plasmids are constructed by cloning the gene for over-expression downstream of the *S. cerevisiae PGK1* or *TDH3* promoter in a 2μ-based vector. Construction of a DNA library is detailed in the Examples. Note that other like *S. cerevisiae* promoters can also be used for overexpression, including *ADH2, PDC1, PGI1*, etc.

[0071] *E. coli* cells may be grown in LB liquid media or on LB agar plates supplemented with ampicillin at 100 μg/ml as needed. Transformation of *E. coli* DH5α is by electrotransformation according to a protocol by Invitrogen (Invitrogen 11319-019). After transformation, the baterial cells are plated on LB plates containing 100 μg/ml ampicillin for selection. Transformation of *S. cerevisiae* was performed using a DMSO-enhanced lithium-acetate procedure as described with the following modifications (Hill et al., 1991). Cells are harvested and initially washed in water. 600 μl of PEG4000 solution is added and 70 μl DMSO is added just prior to heat shocking. Cells are heat-shocked for 15 min at 42°C and the last wash step is skipped. Cells are resuspended in 10 mM TE solution and plated.

[0072] Yeast DNA is isolated using the Easy DNA kit according to manufacturer's protocol (Invitrogen, K1800-01). DNA manipulations and library construction are performed as described in Molecular Cloning: A Laboratory Manual (1989), except otherwise specifically indicated in this disclosure. Plasmids are cured from yeast by growing the strain in rich non-selective media overnight followed by plating on non-selective media. Isolated colonies are replica plated to screen for loss of selective markers. Plasmid rescue is performed by transforming isolated yeast DNA into *E. coli* followed by isolation and characterization. *E. coli* plasmid isolation is accomplished using plasmid spin mini-prep kit according to the manufacturer's manual (Qiagen, 27106). PCR-based chromosomal walking is performed using the Universal GenomeWalker Kit as described (BD Biosciences, K1807-1).

[0073] For the transport assays, cells may be grown in minimal media supplemented with 20 g/L of L-arabinose. Cells are collected in mid-growth and washed twice before suspension in water at 30 mg/ml. Uptake of L-(1-^{14}C)arabinose (54 mCi/mmol, Moravek Biochemicals Inc.) or D-(1-^{14}C) galactose (57 mCi/mmol, Amersham Biosciences) is measured as previously described by Stambuk et al. (2003). Assays are performed in 30 seconds to maintain initial rates after appropriate experiments to ensure uptake is linear for at

least 1 minute. Transport activity is described as nano-moles of labeled sugar transported per mg cell dry weight per minute. Inhibition and competition assays are performed as previously described by Stambuk et al. (2003).

[0074] Sequencing results showed that the KmLAT1 gene contains an ORF of 1668 bp in length. The predicted amino acid sequence of KmLAT1 shares homology with high-affinity glucose transporters, in particular, with *HGT1* from *K. lactis* (Table 4). Km*LAT1* transporter shows a much higher sequence similarity with high-affinity glucose transporters from non-conventional yeast than with transporter proteins encoded by the bacterial araE gene or hexose transporters from *S. cerevisiae* (Fig. 1).

Table 4. Properties and similarities of KmLat1 to other sugar transporters.

gene	Predicted protein (no. of aa/no. of kDa)	pI of protein	Predicted transmem-brane regions	Degree of identity (%) /similarity (%)	Organism	Putative function of gene product
KmLat1	556 / 61.3	8.22	12	-	*K.marxianus*[1]	L-arabinose transporter
KlHgt1	551 / 60.8	5.76	12	77 / 89	*K. lactis*[2]	high affinity glucose transporter
AEL042Cp	547 / 59.8	8.82	12	63 / 82	*A. gossypii*[3]	putative hexose transporter
DEHA0E01738g	545 / 61.1	5.55	12	52 / 70	*D. hansenii*[4]	hexose transporter
CaHgt1	545 / 60.7	8.05	12-13	50 / 71	*C. albicans*[5]	putative hexose transporter
CaHgt2	545 / 60.4	8.48	12-14	51 / 71	*C. albicans*[6]	putative hexose transporter

Accession numbers: 1: Not yet assigned, 2: 1346290, 3: AEL042C, 4: DEHA0E01738g,
5: CAA76466, 6: orf19.3668

[0075] Transmembrane regions predicted for KmLat1 and PgLat2 by the software Tmpred shows 12 transmembrane regions with a larger intercellular loop between regions 6 and 7 (Fig. 2) (See Hofmann et al, 1993), typical of *GAL2* and other yeast sugar transporters having 10-12 transmembrane regions (See e.g., Alves-Araujo et al., 2004; Day et al., 2002; Kruckeberg et al., 1996; Pina et al. ,2004; and Weierstall et al. 1999).

[0076] Like other members of the transporter family, and in particular sugar transporters, KmLat1 and PgLat2 polypeptides are useful in facilitating the uptake of various sugar molecules into the cells. It is envisioned that KmLat1 or PgLat2 polypeptides could be used for other purposes, for example, in analytical instruments or other processes where

uptake of sugar is required. KmLat1 or PgLat2 polypeptides may be used alone or in combination with one or more other transporters to facilitate the movement of molecules across a membrane structure, which function may be modified by one skilled in the relevant art, all of which are within the scope of the present disclosure.

[0077] The KmLAT1 polypeptides include isolated polypeptides having an amino acid sequence as shown below in Example 2; and in SEQ ID NO:2, as well as variants and derivatives, including fragments, having substantial sequence similarity to the amino acid sequence of SEQ ID NO:2 and that retain any of the functional activities of KmLAT1. PgLAT2 polypeptides include isolated polypeptides having an amino acid sequence as shown below in Example 5; and in SEQ ID NO:4, as well as variants and derivatives, including fragments, having substantial sequence similarity to the amino acid sequence of SEQ ID NO:4 and that retain any of the functional activities of PgLAT2. The functional activities of the KmLAT1 or PgLAT2 polypeptides include but are not limited to transport of L-arabinose across cell membrane. Such activities may be determined, for example, by subjecting the variant, derivative, or fragment to a arabinose transport assay as detailed, for example in Example 4.

[0078] Variants and derivatives of KmLAT1 or PgLAT2 include, for example, KmLAT1 or PgLAT2 polypeptides modified by covalent or aggregative conjugation with other chemical moieties, such as glycosyl groups, polyethylene glycol (PEG) groups, lipids, phosphate, acetyl groups, and the like.

[0079] The amino acid sequence of KmLAT1 or PgLAT2 polypeptides is preferably at least about 60% identical, more preferably at least about 70% identical, more preferably still at least about 80% identical, and in some embodiments at least about 90%, 95%, 96%, 97%, 98%, and 99% identical, to the KmLAT1 and PgLAT2 amino acid sequences of SEQ ID NO: 2 and SEQ ID NO: 4, respectively. The percentage sequence identity, also termed homology (see definition above) may be readily determined, for example, by comparing the two polypeptide sequences using any of the computer programs commonly employed for this purpose, such as the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wisconsin), which uses the algorithm of Smith and Waterman, 1981, *Adv. Appl. Math.* 2: 482-489.

[0080] Variants and derivatives of the KmLAT1 or PgLAT2 polypeptides may further include, for example, fusion proteins formed of a KmLAT1 or PgLAT2 polypeptide and

another polypeptide. Fusion protein may be formed between a fragment of the KmLAT1 polypeptide and another polypeptide, such that the fusion protein may retain none or only part of the activities normally performed by the full-length KmLAT1 or PgLAT2 polypeptide. Preferred polypeptides for constructing the fusion protein include those that facilitate purification or oligomerization, or those that enhance KmLAT1 or PgLAT2 stability and/or transport capacity or transport rate for sugars, especially for arabinose. Preferred polypeptides may also include those that gain enhanced transport capability when fused with KmLAT1, PgLAT2 or fragments thereof.

[0081] KmLAT1 or PgLAT2 variants and derivatives may contain conservatively substituted amino acids, meaning that one or more amino acid may be replaced by an amino acid that does not alter the secondary and/or tertiary structure of the polypeptide. Such substitutions may include the replacement of an amino acid, by a residue having similar physicochemical properties, such as substituting one aliphatic residue (Ile, Val, Leu, or Ala) for another, or substitutions between basic residues Lys and Arg, acidic residues Glu and Asp, amide residues Gln and Asn, hydroxyl residues Ser and Tyr, or aromatic residues Phe and Tyr. Phenotypically silent amino acid exchanges are described more fully in Bowie *et al.*, 1990. In addition, functional KmLAT1 or PgLAT2 polypeptide variants include those having amino acid substitutions, deletions, or additions to the amino acid sequence outside functional regions of the protein. Techniques for making these substitutions and deletions are well known in the art and include, for example, site-directed mutagenesis.

[0082] The KmLAT1 or PgLAT2 polypeptides may be provided in an isolated form, or in a substantially purified form. The polypeptides may be recovered and purified from recombinant cell cultures by known methods, including, for example, ammonium sulfate or ethanol precipitation, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography. Preferably, protein chromatography is employed for purification.

[0083] A preferred form of KmLAT1 or PgLAT2 polypeptides is that of recombinant polypeptides expressed by suitable hosts. In one preferred embodiment, when heterologous expression of KmLAT1 or PgLAT2 is desired, the coding sequences of KmLAT1 or PgLAT2 may be modified in accordance with the codon usage of the host. Such modification may result in increase protein expression of a foreign in the host. Furthermore, the hosts may

simultaneously produce other transporters such that multiple transporters are expressed in the same cell, wherein the different transporters may form oligomers to transport the same sugar. Alternatively, the different transporters may function independently to transport different sugars. Such recombinant cells may be useful in crude fermentation processing or in other industrial processing.

[0084] KmLAT1 or PgLAT2 polypeptides may be fused to heterologous polypeptides to facilitate purification. Many available heterologous peptides (peptide tags) allow selective binding of the fusion protein to a binding partner. Non-limiting examples of peptide tags include 6-His, thioredoxin, hemaglutinin, GST, and the OmpA signal sequence tag. A binding partner that recognizes and binds to the heterologous peptide may be any molecule or compound, including metal ions (for example, metal affinity columns), antibodies, antibody fragments, or any protein or peptide that preferentially binds the heterologous peptide to permit purification of the fusion protein.

[0085] KmLAT1 or PgLAT2 polypeptides may be modified to facilitate formation of KmLAT1 or PgLAT2 oligomers. For example, KmLAT1 polypeptides may be fused to peptide moieties that promote oligomerization, such as leucine zippers and certain antibody fragment polypeptides, for example, Fc polypeptides. Techniques for preparing these fusion proteins are known, and are described, for example, in WO 99/31241 and in Cosman et. al., 2001. Fusion to an Fc polypeptide offers the additional advantage of facilitating purification by affinity chromatography over Protein A or Protein G columns. Fusion to a leucine-zipper (LZ), for example, a repetitive heptad repeat, often with four or five leucine residues interspersed with other amino acids, is described in Landschultz et al., 1988.

[0086] It is also envisioned that an expanded set of variants and derivatives of KmLAT1 or PgLAT2 polynucleotides and/or polypeptides may be generated to select for useful molecules, where such expansion is achieved not only by conventional methods such as site-directed mutagenesis but also by more modern techniques, either independently or in combination.

[0087] Site-directed-mutagenesis is considered an informational approach to protein engineering and may rely on high-resolution crystallographic structures of target proteins for specific amino acid changes (van den Burg et al. 1998). For example, modification of the amino acid sequence of KmLAT1 or PgLAT2 polypeptides may be accomplished as is known in the art, such as by introducing mutations at particular locations by oligonucleotide-

directed mutagenesis Site-directed-mutagenesis may also take advantage of the recent advent of computational methods for identifying site-specific changes for a variety of protein engineering objectives (Hellinga, 1998).

[0088] The more modern techniques include, but are not limited to, non-informational mutagenesis techniques (referred to generically as "directed evolution"). Directed evolution, in conjunction with high-throughput screening, allows testing of statistically meaningful variations in protein conformation (Arnold, 1998). Directed evolution technology may include diversification methods similar to that described by Crameri et al. (1998), site-saturation mutagenesis, staggered extension process (StEP) (Zhao et al., 1998), and DNA synthesis/reassembly (U.S. Patent 5,965,408).

[0089] Fragments of the KmLAT1 or PgLAT2 polypeptide may be used, for example, to generate specific anti- KmLAT1 antibodies. Using known selection techniques, specific epitopes may be selected and used to generate monoclonal or polyclonal antibodies. Such antibodies have utility in the assay of KmLAT1 or PgLAT2 activity as well as in purifying recombinant KmLAT1 or PgLAT2 polypeptides from genetically engineered host cells.

[0090] The disclosure also provides polynucleotide molecules encoding the KmLAT1 or PgLAT2 polypeptides discussed above. *KmLAT1* or *PgLAT2* polynucleotide molecules include polynucleotide molecules having the nucleic acid sequence shown in SEQ ID NO:1 and SEQ ID NO:3, respectively; polynucleotide molecules that hybridize to the nucleic acid sequence of SEQ ID NO:1 and SEQ ID NO:3, respectively, under high stringency hybridization conditions (for example, 42°, 2.5 hr., 6X SCC, 0.1%SDS); and polynucleotide molecules having substantial nucleic acid sequence identity with the nucleic acid sequence of SEQ ID NO:1 and SEQ ID NO:3, respectively. It will be appreciated that such polynucleotide molecules also broadly encompass equivalent substitutions of codons that may be translated to produce the same amino acid sequences, truncated fragments of the polynucelotide molecues, and polynucleotide molecules with a high incidence of homology, such as 90%, 95%, 96%, 97%, 98%, or 99% or more homology with respect to what is disclosed.

[0091] The *KmLAT1* or *PgLAT2* polynucleotide molecules of the disclosure are preferably isolated molecules encoding the KmLAT1 or PgLAT2 polypeptide having an amino acid sequence as shown in SEQ ID NO:2 and SEQ ID NO:4, respectively, as well as derivatives, variants, and useful fragments of the *KmLAT1* or *PgLAT2* polynucleotide. The

KmLAT1 or *PgLAT2* polynucleotide sequence may include deletions, substitutions, or additions to the nucleic acid sequence of SEQ ID NO:1 and SEQ ID NO:3, respectively.

[0092] The *KmLAT1* or *PgLAT2* polynucleotide molecule may be cDNA, chemically synthesized DNA, DNA amplified by PCR, RNA, or combinations thereof. Due to the degeneracy of the genetic code, two DNA sequences may differ and yet encode identical amino acid sequences. The present disclosure thus provides an isolated polynucleotide molecule having a *KmLAT1* or *PgLAT2* nucleic acid sequence encoding *KmLAT1* or *PgLAT2* polypeptide, wherein the nucleic acid sequence encodes a polypeptide having the complete amino acid sequences as shown in SEQ ID NO:2 and SEQ ID NO:4, respectively, or variants, derivatives, and fragments thereof.

[0093] The *KmLAT1* or *PgLAT2* polynucleotides of the disclosure have a nucleic acid sequence that is at least about 60% identical to the nucleic acid sequence shown in SEQ ID NO:1 and SEQ ID NO:3, respectively, in some embodiments at least about 70% identical to the nucleic acid sequence shown in SEQ ID NO:1 and SEQ ID NO:3, respectively, at least about 80% identical to the nucleic acid sequence shown in SEQ ID NO:1 and SEQ ID NO:3, respectively, and in other embodiments at least about 90% -95%, 96%, 97%, 98%, 99%% identical to the nucleic acid sequence shown in SEQ ID NO:1 and SEQ ID NO:3, respectively. Nucleic acid sequence identity is determined by known methods, for example by aligning two sequences in a software program such as the BLAST program (Altschul, S.F et al. (1990) J. Mol. Biol. 215:403-410, from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

[0094] The *KmLAT1* or *PgLAT2* polynucleotide molecules of the disclosure also include isolated polynucleotide molecules having a nucleic acid sequence that hybridizes under high stringency conditions (as defined above) to a the nucleic acid sequence shown in SEQ ID NO:1 and SEQ ID NO:3, respectively. Hybridization of the polynucleotide is to at least about 15 contiguous nucleotides, or at least about 20 contiguous nucleotides, and in other embodiments at least about 30 contiguous nucleotides, and in still other embodiments at least about 100 contiguous nucleotides of the nucleic acid sequence shown in SEQ ID NO:1 and SEQ ID NO:3, respectively.

[0095] Useful fragments of the *KmLAT1* or *PgLAT2* polynucleotide molecules described herein, include probes and primers. Such probes and primers may be used, for example, in PCR methods to amplify and detect the presence of *KmLAT1* or *PgLAT2*

polynucleotides *in vitro*, as well as in Southern and Northern blots for analysis of KmLAT1 or PgLAT2. Cells expressing the *KmLAT1* or *PgLAT2* polynucleotide molecules may also be identified by the use of such probes. Methods for the production and use of such primers and probes are known. For PCR, 5' and 3' primers corresponding to a region at the termini of the *KmLAT1* or *PgLAT2* polynucleotide molecule may be employed to isolate and amplify the *KmLAT1* or *PgLAT2* polynucleotide using conventional techniques.

[0096] Other useful fragments of the *KmLAT1* or *PgLAT2* polynucleotides include antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence capable of binding to a target KmLAT1 or PgLAT2 mRNA (using a sense strand), or DNA (using an antisense strand) sequence.

[0097] The present disclosure also provides vectors containing the polynucleotide molecules, as well as host cells transformed with such vectors. Any of the polynucleotide molecules of the disclosure may be contained in a vector, which generally includes a selectable marker and an origin of replication, for propagation in a host. The vectors may further include suitable transcriptional or translational regulatory sequences, such as those derived from a mammalian, fungal, bacterial, viral, or insect genes, operably linked to the *KmLAT1* or *PgLAT2* polynucleotide molecule. Examples of such regulatory sequences include transcriptional promoters, operators, or enhancers, mRNA ribosomal binding sites, and appropriate sequences which control transcription and translation. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the DNA encoding the target protein. Thus, a promoter nucleotide sequence is operably linked to a *KmLAT1* or *PgLAT2* DNA sequence if the promoter nucleotide sequence directs the transcription of the *KmLAT1* or *PgLAT2* sequence.

[0098] Selection of suitable vectors for the cloning of *KmLAT1* or *PgLAT2* polynucleotide molecules encoding the *KmLAT1* or *PgLAT2* polypeptides of this disclosure depends upon the host cell in which the vector will be transformed, and, where applicable, the host cell from which the target polypeptide is to be expressed. Suitable host cells for expression of *KmLAT1* or *PgLAT2* polypeptides include prokaryotes, yeast, and higher eukaryotic cells, each of which is discussed below. Selection of suitable combinations of vectors and host organisms is a routine matter from a perspective of skill.

[0099] The *KmLAT1* or *PgLAT2* polypeptides to be expressed in such host cells may also be fusion proteins that include sequences from other proteins. As discussed above, such

regions may be included to allow, for example, enhanced functionality, improved stability, or facilitated purification of the *KmLAT1* or *PgLAT2* polypeptide. For example, a nucleic acid sequence encoding a peptide that binds strongly to arabinose may be fused in-frame to the transmembrane sequence of the *KmLAT1* or *PgLAT2* polypeptides so that the resulting fusion protein binds arabinose and transports the sugar across the cell membrane at a higher rate than the *KmLAT1* or *PgLAT2* transporter.

[00100] Suitable host cells for expression of target polypeptides include prokaryotes, yeast, and higher eukaryotic cells. Suitable prokaryotic hosts to be used for the expression of these polypeptides include bacteria of the genera *Escherichia, Bacillus, and Salmonella,* as well as members of the genera *Pseudomonas, Streptomyces,* and *Staphylococcus.*

[00101] Expression vectors for use in prokaryotic hosts generally comprise one or more phenotypic selectable marker genes. Such genes encode, for example, a protein that confers antibiotic resistance or that supplies an auxotrophic requirement. A wide variety of such vectors are readily available from commercial sources. Examples include pSPORT vectors, pGEM vectors (Promega, Madison, WI), pPROEX vectors (LTI, Bethesda, MD), Bluescript vectors (Stratagene), and pQE vectors (Qiagen).

[0100] *KmLAT1* or *PgLAT2* may also be expressed in yeast host cells from genera including *Saccharomyces, Pichia,* and *Kluveromyces.* Preferred yeast host is *S. cerevisiae.* Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid for high copy vectors and a CEN sequence for a low copy number vector. Other sequences on a yeast vector may include an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Vectors replicable in both yeast and *E. coli* (termed shuttle vectors) are preferred. In addition to the above-mentioned features of yeast vectors, a shuttle vector will also include sequences for replication and selection in *E. coli.*

[0101] Insect host cell culture systems may also be used for the expression of *KmLAT1* or *PgLAT2* polypeptides. The target polypeptides are preferably expressed using a baculovirus expression system, as described, for example, in the review by Luckow and Summers, 1988.

[0102] The choice of a suitable expression vector for expression of *KmLAT1* or *PgLAT2* polypeptides will depend upon the host cell to be used. Examples of suitable

expression vectors for *E. coli* include pET, pUC, and similar vectors as is known in the art. Preferred vectors for expression of the *KmLAT1* or *PgLAT2* polypeptides include the shuttle plasmid pIJ702 for *Streptomyces lividans*, pGAPZalpha-A, B, C and pPICZalpha-A, B, C (Invitrogen) for *Pichia pastoris*, and pFE-1 and pFE-2 for filamentous fungi and similar vectors as is known in the art. The vectors preferred for expression in *S. cerevisiae* are listed in Table 2.

[0103] Modification of a *KmLAT1* or *PgLAT2* polynucleotide molecule to facilitate insertion into a particular vector (for example, by modifying restriction sites), ease of use in a particular expression system or host (for example, using preferred host codons), and the like, are known and are contemplated for use. Genetic engineering methods for the production of *KmLAT1* or *PgLAT2* polypeptides include the expression of the polynucleotide molecules in cell free expression systems, in host cells, in tissues, and in animal models, according to known methods.

[0104] This disclosure also provides reagents, compositions, and methods that are useful for analysis of *KmLAT1* or *PgLAT2* activity and for assessing the amount and rate of arabinose transport.

[0105] The KmLAT1 or PgLAT2 polypeptides of the present disclosure, in whole or in part, may be used to raise polyclonal and monoclonal antibodies that are useful in purifying *KmLAT1* or *PgLAT2*, or detecting *KmLAT1* or *PgLAT2* polypeptide expression, as well as a reagent tool for characterizing the molecular actions of the *KmLAT1* or *PgLAT2* polypeptide. Preferably, a peptide containing a unique epitope of the *KmLAT1* or *PgLAT2* polypeptide is used in preparation of antibodies, using conventional techniques. Methods for the selection of peptide epitopes and production of antibodies are known. See, for example, *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), 1988 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Kennet *et al.* (eds.), 1980 Plenum Press, New York.

[0106] Agents that modify, for example, to increase or decrease, *KmLAT1* or *PgLAT2* transport of arabinose or other sugars may be identified by the transport assay described in Example 4, for example. Performing the transport assay in the presence or absence of a test agent permits screening of such agents.

[0107] The KmLAT1 or PgLAT2 transport activity is determined in the presence or absence of a test agent and then compared. For instance, a lower *KmLAT1* transport activity in the presence of the test agent, than in the absence of the test agent, indicates that the test

agent has decreased the activity of the *KmLAT1*. Stimulators and inhibitors of *KmLAT1* or *PgLAT2* may be used to augment, inhibit, or modify *KmLAT1* or *PgLAT2* transport activity, and therefore may have potential industrial uses as well as potential use in further elucidation of the molecular actions of *KmLAT1* or *PgLAT2*.

[0108] The KmLAT1 or PgLAT2 polypeptide of the disclosure is an effective arabinose transporter. In the methods of the disclosure, the sugar transporting effects of *KmLAT1* or *PgLAT2* are achieved by mixing cells expressing *KmLAT1* or *PgLAT2* with pure sugar or sugar-containing biomass. *KmLAT1* or *PgLAT2* may also be used in a cell-free system. *KmLAT1* or *PgLAT2* may be used under other conditions, for example, at elevated temperatures or under acidic pH. Other methods of using *KmLAT1* or *PgLAT2* to transport sugar, especially arabinose, for fermentation, are envisioned to be within the scope of what is disclosed. *KmLAT1* or *PgLAT2* polypeptides may be used in any known application currently utilizing a sugar transporter, all of which are within the scope of this disclosure.

[0109] It is shown in this disclosure that Gal2p is an effective L-arabinose transporter at high concentrations of arabinose, whereas *KmLAT1* or *PgLAT2* may be more effective at different concentrations of L-arabinose. Combination of the Gal2p and the two new transporters from non-conventional yeast may be employed to provide complementary transport into *S. cerevisiae* of L-arabinose down to very low residual concentration of arabinose.

[0110] It is shown here that combinatorial expression of Gal2p, KmLAT1 and PgLAT2 may enhance the overall rate and extent of arabinose utilization by recombinant *S. cereviciae* cells expressing these transporters. As shown in Example 8, the doubling time for *S. cereviciae* strain expressing both PgLAT2 and Gal2p is shorter than *S. cereviciae* cells expressing Gal2p alone (15 hours *vs.* 19 hours), suggesting that L-arabinose uptake may be enhanced by the synergistic effect of PgLAT2 and Gal2p in these cells. Moreover, the PgLAT2 expressing strain appears to grow to a higher overall optical density at saturation, suggesting that this strain was able to utilize the carbon source (L-arabinose) in the medium more completely. This hypothesis is supported by HPLC analysis of the final culture media (Table 5) which indicates that there is significantly less residual L-arabinose in the culture of cells expressing Gal2p and PgLAT2 than in the culture of those expressing Gal2p alone. Thus, heterologous expression of either or both KmLAT1 and PgLAT2 in *S. cereviciae* may enhance arabinose utilization by facilitating arabinose transport when the concentration of arabinose is relatively low.

Table 5 Doubling times and HPLC Measurement of Residual Arabinose Concentration in Cultures Described in Figure 11.

Flask	Transporters Expressed	Doubling Time (hours)	Final OD_{600}	L-arabinose (g/L) by HPLC
1	Gal2p only	19.2	0.72	0.68
2		18.6	0.72	0.67
3	Gal2p + PgLat2	15.0	0.85	0.49
4		14.8	0.85	0.48

*starting L-arabinose concentration 1.89 g/L and media without L-arabinose had an undetectable level (<0.1 g/L). ND = not determined.

[0111] L-arabinose metabolism in bacteria involves three enzymes: L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), and L-ribulose-5-p 4-epimerase (*araD*), which may be collectively referred to as the "araBAD" proteins in this disclosure. The genes encoding these three enzymes may be referred to as the "araBAD" genes in this disclosure. The combined action of these three bacterial proteins convert L-arabinose to Xylulose-5-phosphate (See Fig. 3). *S. cerevisiae* contains the pathway to utilize and ferment the final product xylulose-5-phosphate and produce ethanol under certain conditions (see Fig. 3).

[0112] *S. cerevisiae* strain to be used to construct an arabinose fermenting yeast strain preferably possesses Gal^+ phenotype. A Gal^+ strain is likely to express galactose permease which may facilitate the uptake of arabinose by *S. cerevisiae*.

[0113] *S. cerevisiae* typically possesses endogenous aldose reductase ("AR") activity, which may divert arabinose to a pathway different from the one that may lead to the production of ethanol through the action of the bacterial araBAD proteins. Moreover, the arabitol generated by the AR protein may inhibit the isomerase encoded by *araA*. In order to increase the overall yield of ethanol from arabinose, it is preferable to use an AR-deficient strain to construct the arabinose fermenting yeast of the present disclosure. The AR-deficient strain may be obtained by screening for spontaneous mutations, or preferably by targeted gene disruption or mutation. An example of such gene disruption is detailed in Example 10.

[0114] As shown in Fig. 3, the engineered pathway utilizing bacterial araBAD converts L-arabinose to xylulose-5-P that *S. cerevisiae* can convert to ethanol using endogenous enzymatic activities. It is thus desirable to ensure that the arabinose metabolic pathway starting from xylulose to ethanol remains intact in the AR-deficient strain. This may be tested by comparing the growth of the AR-deficient strain with its parental strain on glucose or xylulose. If both strains proliferate at about the same rate on glucose or xylulose,

it is likely that the AR gene disruption event has not negatively impacted the catabolism of glucose or xylulose.

[0115] The present disclosure also provides a new method to measure arabinose uptake by yeast cells. Traditionally, L-arabinose transport is measured by using radio-labeled substrate. Since aldose reductase, which converts L-arabinose to arabitol, is cytosolic, it is possible to use the formation of new arabitol as an indicator of arabinose uptake. Higher levels of arabitol indicates higher uptake of L-arabinose. To confirm the validity of this method, L-arabinose transport was measured using the traditional ^{14}C-labelled L-arabinose in various yeast strains with different levels of arabitol formation. These experiments show that the level of arabitol formation corresponds well with the level of L-arabinose uptake.

[0116] Using this method, several high arabitol producing strains have been isolated, including two *gal80* mutants and two otherwise wildtype strains, which have 3 to 4 folds higher L-arabinose and D-galactose transport activity than the BFY001 originally used to construct the arabinose fermenting strains. Bacterial genes encoding the araBAD proteins may be introduced into these strains to achieve higher rate of arabinose uptake and thus higher overall yield. This result also validates the indirect screening method for strains with higher arabinose transport activity.

[0117] Although the present disclosure teaches the introduction of foreign genes such as *E. coli araBAD* genes, into yeast cells, genes from other species encoding proteins that perform the same or similar function as the *E. coli araBAD* proteins, i.e., converting L-arabinose into various intermediates and eventually into ethanol, may be used in place of the *E. coli araBAD* genes (See e.g., Becker and Boles, 2003, using *araA* from *Bacillus subtilis*). The DNA of the foreign genes may be present in a host cell at one copy, or preferably, in more than one copy. The foreign genes may be under control of a constitutive promoter or an inducible promoter.

[00102] The foreign genes may be present as plasmids or minichromosomes in the host yeast cells, or alternatively, the plasmids carrying the foreign genes may be engineered so that the foreign genes are integrated into the chromosomes of the host through genetic recombination. In the latter case, the foreign tends to be maintained after generations, even when the host cells are grown in rich media where no selective pressure is present. By contrast, in the former case where the genes remain on a vector, the genes may be lost after a few generations. Under those circumstances, the host yeast cells are preferably grown in a

minimum media supplemented with appropriate amino acids or purine or pyrimidine bases so that a selective pressure helps maintain the plasmids.

[0118] Cell-free or whole-cell fermentation may be used to convert arabinose to ethanol. In the whole cell fermentation process, the transformants may be grown on minimum media with appropriate supplementation to maintain the plasmids. The transformant cells are preferably grown on galactose to induce the expression of galactose permease. More preferably, the cells are grown on both arabinose and galactose before the fermentation assays. In addition, transformant cells can be grown on both arabinose and glucose before the fermentation assays (see Fig. 19).

[0119] In the cell-free fermentation system, cells are harvested and the cells are lysed to release enzymes for the conversion of the sugar to ethanol. One bottleneck for a whole-cell fermentation system is the uptake of arabinose by the cells, which may explain its lower overall yield of ethanol than the cell-free system. However, whole-cell fermentation is preferred because it is easier to perform. In a whole-cell fermentation system, the cells may be mixed directly with the biomass or other substrates, requiring no extra steps of cell-handling.

[0120] In a preferred embodiment, the various arabinose metabolic pathways disclosed here may be introduced into a *S. cerevisiae* strain that have been modified to facilitate its arabinose uptake. Such strain may include but are not limited to strains that express both the Gal2p and one or two of the novel arabinose transporters similar to the ones disclosed here. The expression levels of the array of arabinose transporters may be fine-tuned such that they are commensurate with the rate of arabinose metabolism inside the engineered yeast cells. Most preferably, the expression levels of the transporters may be linked to the arabinose metabolic rate in each cell, such that the arabinose is taken in more rapidly by those cells that convert arabinose to ethanol more efficiently.

[0121] The examples herein illustrate the present instrumentalities by way of illustration, and not by limitation. The chemicals, biological agents and other ingredients are presented as typical components or reactants, and the procedures described herein may represent but one of the typical ways to accomplish the goal of the particular experiment. It is understood that various modification may be derived in view of the foregoing disclosure without departing from the spirit of the present disclosure.

Example 1—Cloning of the new transporter gene *KmLAT1*

[0122] A *K. marxianus* genomic library was constructed in our yeast vector pBFY13 which contains the yeast 2μ origin of replication, a *URA3* selection cassette, and a *BamHI* site located between the *PGK1* promoter and *GAL10* terminator. After partial digestion of 200 μg of genomic DNA with *Sau3AI* restriction enzyme, fragments of 2-8 kb in length were gel-isolated and ligated into the *BamHI* site of pBFY013. This ligation reaction was then transformed into *E. coli* and plated for recovery. Plate counts produced ~3000 cfu's/10 μl of transformed cells and the plasmid DNA from 24 colonies was screened for presence of insert revealing 22 of 24 transformants had an insert ranging from 1 kb to ~8 kb giving an average insert size of 3.2 kb. The transformed cells were scraped from the plates, DNA recovered, and 5 μl was transformed into competent BFY518 cells. The strain, BFY518, was cured of the *GAL2* over-expression plasmid negating its ability to form colonies on agar plates containing L-arabinose as the sole carbon source enabling restoration of colony formation by complementation with a heterologous L-arabinose transporter. To count the number of transformed yeast cells, 10 μl of the yeast library transformation were plated onto minimal glucose media yet the colonies were so dense that only an estimate of ~5000 colonies was possible. The rest of the transformation mix (~140 μl) was plated onto minimal media containing 2% L-arabinose for selection from which a small amount of background growth was noticed. The plates were then replica plated to fresh L-arabinose minimal media. The total number of cells plated for selection represented ~280,000 transformants representing ~8 fold coverage of the 10.7 mb *K. marxianus* genome (See Dujon et al., 2004). Two colonies grew on the replica plates and the plasmid DNA was rescued and re-transformed into BFY518 allowing growth once again on L-arabinose confirming that the *K. marxianus* genomic insert carried on these plasmids was responsible for growth. Restriction analysis suggested both plasmids harbored the same insert of approximately 8.8 kb in size.

Example 2---Sequence Analysis of the *KmLAT1* gene

[0123] Sequencing results showed that both plasmids had identical inserts of 8838 kb containing two ORFs on the 5' end of the insert. Both of these ORFs showed strong homology to yeast sugar transporters. One transporter ORF was interrupted by a fragment of an unrelated ORF suggesting that recombination of fragments during ligation into the vector occurred in library construction (Fig. 4). Recombination of library fragments during ligation into the vector was shown by PCR walking experiments performed on *K. marxianus* genomic DNA. Walking was performed out of the transporter in a 5' direction and additional transporter sequence including the start codon was recovered rather than the additional sequence from the unrelated ORF. The uninterrupted transporter ORF, termed Km*LAT1*, was recovered twice more in another subsequent library screening. This ORF was 1668 bp in length and shared homology with high-affinity glucose transporters in particular, *HGT1* from *K. lactis* (Table 4) and showed a much closer association with high-affinity glucose transporters from non-conventional yeasts than the bacterial araE genes or *S. cerevisiae* hexose transporters (Fig. 1).

[0124] Transmembrane region prediction by the software Tmpred shows 12 transmembrane regions with a larger intercellular loop between regions 6 and 7 (Fig. 2) (See Hofmann et al, 1993), typical of *GAL2* and other yeast sugar transporters having 10-12 transmembrane regions (See e.g., Alves-Araujo et al., 2004; Day et al., 2002; Kruckeberg et al., 1996; Pina et al. ,2004; and Weierstall et al. 1999).

Example 3--KmLat1 Expressed in *S. cerevisiae* Enables Growth on Arabinose

[0125] The coding sequence of Km*LAT1* was isolated by PCR from genomic DNA of *K. marxianus* and cloned into a yeast 2μ plasmid under control of the *PGK1* promoter of *S. cerevisiae*. This construct was transformed into a *GAL2* deleted strain of *S. cerevisiae* adapted to L-arabinose. Briefly, cells are grown in appropriate selective glucose minimal media until saturation then washed and diluted to a starting OD_{600} of 0.2 in minimal media supplemented with 2% L-arabinose. Cultures are incubated until exponential growth is observed then the cultures are diluted twice into the same media for continued growth to establish the final L-arabinose utilizing adapted strain which is purified on streak plates. Control plasmids carrying the yeast GAL2 gene and an empty vector were also used to transform yeast cells.

[0126] Yeast cells with a 2μ plasmid carrying the Km*LAT1* or GAL2 gene or cells with an empty 2μ plasmid were grown with shaking in liquid minimum media containing 2% L-arabinose as the sole carbon source. The OD_{600} of each culture was measured and monitored by 140 hours. Growth curve results show that KmLat1 is sufficient to support growth on L-arabinose when compared to cells harboring the empty vector which does not show any signs of growth (Fig. 5). This result confirms that the KmLat1 gene encodes an arabinose transporter that enables yeast cells to grow on L-arabinose.

Example 4—Comparison of the Arabinose Transport Kinetics between Gal2 and KmLat1 expressed in *S. cerevisiae*

[0127] The transport characteristics of the KmLat1 and the Gal2 transporters expressed in *S. cerevisiae* were compared. Both transporters were expressed in a host background adapted for growth on L-arabinose in which the endogenous copy of *GAL2* had been entirely replaced with a *HIS3* selection marker. The KmLat1 transporter showed a low-affinity transporter having a K_m = 230 mM and a V_{max} = 55 nmol/mg·min (Fig. 6A). This is in contrast to the high-affinity active transport activity induced in the wild type *K. marxianus* when grown on 2% L-arabinose (Fig. 6B). These results suggest there are at least 2 transporters in *K. marxianus* that may transport L-arabinose but just the high-affinity activity is induced in the wild type when grown on 2% L-arabinose. Inhibition experiments showed that when KmLat1 is expressed in *S. cerevisiae* it is not significantly inhibited by protonophores such as NaN_3, DNP, and CCP. Neither is KmLat1 inhibited by H+-adenosine triphosphatase (ATPase) inhibitors such as DESB and DCCD (Table 6). This is in contrast to the transport activity in wild type *K. marxianus*, suggesting that KmLat1 is a facilitated diffusion permease similar to the Gal2 permease. Competition experiments showed that KmLat1 is out-competed by glucose, galactose, xylose, and maltose when expressed in *S. cerevisiae* (Table 6).

Table 6. Effect of Inhibitors or Competing Sugars on the Rate of L-Arabinose Transport in L-Arabinose-Grown *S. cerevisiae* Expressing *GAL2* or Km*LAT1*

Inhibitor or Competing Sugar	Concentration (m*M*)	Relative L-arabinose transport (%)	
		Gal2	KmLat1
None	NA	100[a]	100[c]
NaN_3	10	66	11
CCCP	5	46	61
DCCD	5	69	55
DNP	5	72	75
DESB	5	81	100
None	NA	100[b]	100[d]
Glucose	900	10	17
Galactose	900	3	23
Xylose	900	25	25
Maltose	450	ND	38

[a] Uptake rate was 66.0 nmol mg^{-1} min^{-1} determined with 118 m*M* labeled L-arabinose.
[b] Uptake rate was 18.9 nmol mg^{-1} min^{-1} determined with 30 m*M* labeled L-arabinose.
[c] Uptake rate was 7.7 nmol mg^{-1} min^{-1} determined with 118 m*M* labeled L-arabinose.
[d] Uptake rate was 3.6 nmol mg^{-1} min^{-1} determined with 30 m*M* labeled L-arabinose.
ND, Not Done.

[0128] Transport kinetics of *S. cerevisiae* BFY597 over-expressing the Gal2 permease grown on 2% L-arabinose showed a K_m of 550 mM and a V_{max} of 425 nmol/mg·min for L-arabinose transport (Fig. 6A). Inhibition assays showed a reduction but not a complete inhibition of transport suggestive of facilitated diffusion transport (Table 6). Competition studies showed that glucose, galactose, and xylose significantly reduced L-arabinose transport indicating that these sugars are preferentially transported over L-arabinose (Table 6). The kinetics of galactose transport were also measured in this strain and indicate that Gal2p has a K_m of 25 m*M* and a V_{max} of 76 nmol/mg·min for galactose transport (data not shown) demonstrating a higher affinity for galactose that would out-compete L-arabinose for transport.

Example 5—Cloning of the New Transporter Gene *PgLAT2*

[0129] Wildtype *Pichia guilliermondii* NRRL Y-2075 was obtained from the Agricultural Research Service Culture Collection (NRRL). *Pichia guilliermondii* cells were

grown in minimal media supplemented with 2% L-arabinose, galactose, or xylose. Cells were collected in mid-growth and washed twice in water before suspension in water at about 30 mg/ml. RNA was extracted from the cells using the acid phenol method (Ausubel, et al., Short Protocols in Molecular Biology, John Wiley and Sons, 1999). Briefly, approximately 15 mL of fresh culture was added to about 25 mL of crushed ice and centrifuged at 4° C for 5 min at 3840 x g. Cells were washed twice with cold DEPC-treated water, and the pellets were frozen at -80° C. After the pellets were resuspended in 400 ul TES (10 mM Tris HCl, pH 7.5, 5 mM EDTA, 0.5% SDS), 400 ul of acid phenol was added. The samples were vortexed vigorously for 10 sec, followed by incubation for 30-60 min at 65° C with occasional vortexing. The tubes with the samples were then chilled on ice and spun for 5 min at 4° C. The aqueous phase was removed and re-extracted with chloroform. The aqueous phase was then ethanol precipitated using 0.1 volume of 3 M sodium acetate (pH 5.3) and two volumes of 100% ethanol. The pellet was washed using 80% ethanol, dried, and resuspended in 50 ul DEPC H_2O. Total RNA concentration was quantitated by measuring the OD_{260} and visualized on agarose gels.

[0130] RNA purification, synthesis of cDNA, and differential display were performed at GenHunter Corporation according to standard techniques. DNA Bands showing higher levels of expression from arabinose-grown cells relative to xylose- or galactose-grown cells were reamplified using the differential display amplification primers. Direct sequencing was performed on the PCR products using the GenHunter arbitrary primers. In cases that did not yield clean sequence, the amplification products were cloned in the TOPO-TA vector pCR2.1 (Invitrogen) and individual clones were sequenced. Sequences were then compared to the databases using BLASTX analysis and those that showed similarity to known transporters or transporter-like proteins were examined further. One of these sequences led to the identification of a novel transporter gene, *PgLAT2* from *Pichia guilliermondii*. *PgLAT2* gene has an ORF of 1617 nucleotides encoding a protein with a predicted length of 539 amino acids (Figure 7).

Example 6—Characteristics of Sugar Transport by *Pichia guilliermondii*

[0131] The induction of L-arabinose transport in wild type *P. guilliermondii* was examined. Wildtype *Pichia guilliermondii* cells were grown in minimal media supplemented with 2% L-arabinose, galactose, or xylose while BFY605 cells were grown in the same media supplemented with 0.2% L-arabinose. Cells were collected in mid-growth and washed twice

in water before suspension in water at about 30 mg/ml. Uptake of L-(1-^{14}C)arabinose (54 mCi/mmol, Moravek Biochemicals Inc.), D-(1-^{14}C)galactose (57 mCi/mmol, Amersham Biosciences), or D-(1-^{14}C)xylose (53 mCi/mmol, Moravek Biochemicals Inc.) was measured as previously described (Stambuk, Franden et al. 2003). Assays were performed in 5, 10, or 30 second periods to maintain initial rates. Appropriate experiments ensured uptake was linear for at least 1 minute. Transport activity was described as nmoles of labeled sugar transported per mg cell dry weight per minute. Inhibition and competition assays were performed as previously described (Stambuk, Franden et al. 2003).

[0132] Cells grown on L-arabinose were able to transport L-arabinose whereas cells grown on galactose or xylose were not able to transport L-arabinose. Additionally, xylose transport was about double in cells grown in L-arabinose media compared to cells grown in xylose media. Galactose was transported at the same rate independent of growth substrate (Fig. 8). Transport competition between L-arabinose and xylose was also examined. Uptake of labeled L-arabinose was reduced by 96% when 100x un-labeled xylose was included in the transport assay whereas uptake of labeled xylose was only reduced by 16% when 100x un-labeled L-arabinose was included in the assay (Fig. 9). This data suggests that in *P. guilliermondii*, growth on L-arabinose induces expression of a specific transport system capable of transporting L-arabinose and xylose. Additionally, this system preferentially transports xylose at the expense of L-arabinose if both sugars are present and has a higher transport velocity for xylose than the transport system induced when grown on xylose. By contrast, transport activity for L-arabinose is not induced when grown on xylose.

Example 7—Arabinose Transport Kinetics of *PgLAT2* expressed in *S. cerevisiae*

[0133] The L-arabinose transport characteristics of the *PgLAT2* transporter expressed in *S. cerevisiae* grown on 0.2% L-arabinose medium showed the same L-arabinose transport characteristics as wildtype *P. guilliermondii* (Fig. 10). The *PgLAT2* transporter when expressed in *S. cerevisiae* has a K_m = 0.07 mM and V_{max} = 18 nmol/mg-min. Inhibition experiments showed significant inhibition of transport by protonophores (NaN_3, DNP, and CCP) and H+-adenosine triphosphatase (ATPase) inhibitors (DESB and DCCD) similar to the inhibition observed in wildtype *P. guilliermondii* (Table 7). Competition experiments showed that L-arabinose uptake by the *PgLAT2* transporter was inhibited by glucose, galactose, xylose and to a lesser extent by maltose (Table 7).

Table 7
Effect of Inhibitors or Competing Sugars on the Rate of L-Arabinose Transport in L-Arabinose-Grown *P. guilliermondii* Y-2075 and *S. cerevisiae* BFY605

Inhibitor or Competing Sugar	Concentration (mM)	Relative L-arabinose transport	
		P. guilliermondii	*S. cerevisiae (PgLAT2* transporter*)*
None [a]	-	100	100
NaN_3	10	1	16
DNP	5	0	4
CCCP	5	0	2
DCCD	5	22	36
DESB	5	8	1
None [b]	-	100	100
Glucose	120	ND	17
Galactose	120	ND	20
Xylose	120	4	0
Maltose	120	ND	30

[a] Rate of L-arabinose transport was 11.2 nmol mg^{-1} min^{-1} for *P. guilliermondii* and 10.4 nmol mg^{-1} min^{-1} for *S. cerevisiae* (PgLAT2 transporter) determined with 0.33 mM labeled L-arabinose.
[b] Rate of L-arabinose transport was 14.2 nmol mg^{-1} min^{-1} for *P. guilliermondii* and 14.4 nmol mg^{-1} min^{-1} for *S. cerevisiae* (PgLAT2 transporter) determined with 1.2 mM labeled L-arabinose.

[0134] The transport activities, inhibition profiles, and competition rates with respect to xylose of wildtype *P. guilliermondii* and of the *PgLAT2* transporter expressed in *S. cerevisiae* are identical suggesting that *P. guilliermondii* has a single, high affinity, active transporter charged with uptake of L-arabinose. There are no L-arabinose transport activities that are unaccounted which suggests the presence of a single L-arabinose transporter in *P. guilliermondii*.

Example 8—Synergistic Effect on Growth Rate and Sugar Utilization by *S. cerevisiae* Expressing Gal2p and the New Transporter Proteins-PgLat2 and KmLat1

[0135] To determine the complementary effects on arabinose transport by the three transporters, namely, Gal2p, PgLat2 and KmLat1, yeast strains were constructed with appropriate selection markers to allow different pathway and transporter combinations to be expressed. All possible transporter combinations were generated by introducing transporter expression plasmids for PgLat2 and KmLat1 (or empty vectors) into *S. cerevisiae* strains expressing the bacterial genes *araA*, *araB* and *araD* (See e.g., Becker and Boles). All strains expressing Gal2p (due to the *gal80Δ* genotype), plus or minus other transporters, were able to grow on 2% or 0.2% L-arabinose after extensive lag times (a process termed "adaptation."). A relatively low concentration of L-arabinose (0.2%) was used in this experiment as strain differences are more pronounced at this concentration. Once "adapted" to growth on 0.2% L-arabinose, the strains were able to grow more quickly and growth curves for each transporter combination were generated.

[0136] Figure 11 shows a comparison of shake flask growth curves for four strains on 0.2% L-arabinose, all of which express Gal2p (*via* the *GAL80* deletion) in the absence or presence of the novel transporter PgLat2 (also see Table 5). A significant lag time was observed due to their inoculation from stationary cultures. However, once growth initiated, the growth rate was relatively rapid. The doubling time for each culture in the exponential phase of the curve is shown in Table 5. The doubling time for the PgLat2 and Gal2p co-expressing cells was markedly shorter than in the cells expressing only Gal2p (15 hours *vs.* 19 hours). A second observation relates to the overall extent of growth. The PgLat2 expressing strain appeared to grow to a higher overall optical density at saturation, suggesting that this strain was able to utilize the carbon source (L-arabinose) in the medium more completely (Fig. 11).

Example 9—Co-expression of Gal2p with PgLAT2 or KmLAT1 Enables more Complete Utilization of Arabinose by Recombinant *S. cerevisiae*

[0137] Doubling times for the cultures described above in Example 8 were measured in early exponential phase for each culture. Doubling time was measured by the period of time taken for the number of cells to double in a given cell culture (See generally, Guthirie and Fink, 1991). The concentration of remaining L-arabinose at the 276 hour time point was determined by HPLC (for saturated cultures only). The concentration of L-arabinose in the

starting media was about 1.89 g/L and the concentration of L-arabinose in media without L-arabinose had an undetectable level (<0.1 g/L). As shown in Table 5, significantly less residual L-arabinose remained in the culture of cells expressing both Gal2p and PgLAat2 than in the culture of cells expressing Gal2p alone.

Example 10—Construction of *S. cerevisiae* Strain Deficient in Aldose Reductase (AR)

[0138] Based on the sequence of a presumptive AR gene, two oligonucleotide primers were designed and the AR gene along with 600 bp of flanking DNA were cloned by PCR using genomic DNA isolated from yeast as template. Using another set of primers, an AR deletion construct was made in which all the coding sequences of the AR gene were replaced with a restriction enzyme site (*SalI*). The yeast *LEU2* gene was isolated as a *SalI-XhoI* fragment and cloned into the *SalI* site of the AR deletion construct. The DNA fragment containing the *LEU2* gene and AR flanking sequences was used to transform the *leu2*$^-$ yeast strain BFY001. LEU$^+$ transformants were isolated, grown and analyzed by Southern and PCR analysis to confirm that the AR gene in the genome had been deleted and replaced with the *LEU2* gene by homologous recombination.

[0139] One such transformant, designated BFY002, was chosen as a host for further construction of arabinose fermenting yeast strains. Shake-flask experiments were conducted and the results showed that arabitol formation in BFY002 had been reduced to about 50% in comparison with the parental strain BFY001.

[0140] Growth of BFY002 on glucose and xylulose was compared with that of BFY001. Briefly, yeast strains BFY001 and BFY002 were grown in rich medium YPD. Cells were collected by centrifugation and washed with sterile water. The washed cells were suspended in water at the original density. 50 µl of this cell suspension was used to inoculate 5 ml of medium containing yeast nitrogen base ("YNB") supplemented with leucine ("Leu"), tryptophan ("Trp"), histidine ("His") and uracil ("Ura"), plus with 1% glucose or 1% xylulose. The growth of each strain on both media at 30° C with shaking was monitored by measuring the OD_{600} for about 6 generation times. As expected, both strains had much shorter doubling time on glucose than on xylulose. No significant difference in the growth curves was observed between BFY001 and BFY002 regardless of whether glucose or xylulose was used (Fig. 12).

Example 11—Isolation of *E. coli araBAD* genes and *B. subtilis araA* and Introduction into *S. cerevisiae*

[0141] Primers were designed to isolate *E. coli araA* gene as a *BglII* fragment, and the *araB* and *araD* genes were isolated as *BamHI* fragments by PCR from plasmid pZB206. The fragments containing the three genes were cloned into a yeast expression vector pBFY004, which contains *PGK* promoter, *GAL10* terminator, and *TRP1* selection marker. Each plasmid carrying individual gene was then transformed into the yeast strain BFY002 in separate experiments. Transformants carrying individual plasmid were analyzed for the expression levels of each *ara* protein. L-ribulokinase (*araB*) and L-ribulose-5-P-4-epimerase (*araD*) were expressed at a higher level while L-arabinose isomerase (*araA*) was expressed at a lower level.

[0142] In order to introduce all three *ara* genes into the same cell, *URA3* and *HIS3* expression vectors for each *ara* gene were constructed by re-engineering the *TRP1* plasmid, pBFY004. Briefly, the *TRP1* coding sequence was removed and replaced with a *SalI* restriction site to generate a plasmid designated as pBFY011. Other selection markers, *HIS3* or *URA3*, were then cloned into this plasmid. Another strategy for introducing all three ara genes into the same cell was to construct a plasmid carrying all three genes. Briefly this was done by combining each *ara* gene with a different promoter/terminator combination to prevent homologus recombination and thus loop-out of the genes with corresponding loss of function. Primers were designed to clone the *E. coli araD* gene between the *TDH3* promoter and *GAL2* terminator. This expression cassette was then moved to pBFY007 (which already has the *E. coli araA* gene cloned between the *PGK1* promoter and *GAL10* terminator) and designated pBFY051. Similarly primers were designed to clone the *E. coli araB* gene between the *PGI1* promoter and the *PDC1* terminator. This expression cassette was then moved to pBFY051 to creat pBFY090 which now has all three *E. coli ara* genes. The *B. subtilis araA* gene was then isolated using PCR and cloned between the *PGK1* promoter and *GAL10* terminator to replace the *E. coli araA*.The *URA3* gene was isolated as a *SalI* fragment and cloned into the *SalI* site to construct the plasmid pBFY012. Similarly, a *HIS3* expression vector, pBFY013, was constructed by engineering and cloning the *HIS3* gene into pBFY011.

[0143] The engineered *ara* genes were cloned into each of these expression vectors to generate a series of expression vectors carrying each of the *araBAD* genes with either Trp1, Ura3 or His3 markers (Table 3; also see Fig. 13 and Fig. 14). Appropriate combinations of these expression vectors were introduced into the strain BFY002. Similarly, the plasmid

containing all three *ara* genes was introduced into the strain BFY057. The transformants were characterized and assayed for growth and fermentation of arabinose.

Example 12—Determination of the Copy Numbers of Plasmids Carrying *araBAD* in *S. cerevisiae*

[0144] The copy numbers of the three plasmids present in the strain BFY013 (Table 8) transformed as described in Example 11 were determined. On colony of BFY013 was isolated and used to inoculate a flask with yeast minimum medium. The cells were allowed to grown to exponential phase and the cells were harvested by centrifugation. Spheroplasts of the cells were prepared and DNA was extracted from these spheroplasts (See Guthrie and Fink, 1991). *E. coli* strain DH5α cells were then transformed with the extracted yeast DNA. Bacterial transformants were plated out and plasmid DNA in individual colonies was isolated and characterized by restriction digest followed by agarose gel electrophoresis. Assuming that the individual plasmids carrying each of the *araBAD* genes possessed the same capability to transform bacterial cells, the ratio between the copy numbers of each plasmids present in the original yeast cells was estimated based on the number of *E. coli* transformants harboring each plasmid (Table 8).

Table 8. Ratio of the 3 plasmids in BFY013

ara gene	Number
araB	11
araA	4
araD	15

Example 13—Assays of Enzymatic Activities of *araBAD* proteins Expressed in *S. cerevisiae*

[0145] The activities of the three *E. coli* enzymes heterologously expressed in *S. cerevisiae* were measured in the crude extracts of the yeast transformants according to protocol described in Becker and Boles, 2003. The results of these assays are summarized in Table 9. Table 10 compares the enzymatic activities of the two strains used for subsequent fermentation. The enzymatic assays were performed in the presence of absence of 20mM

$MnCl_2$, but it appears that $MnCl_2$ does not have significant effect on the overall results (Table 10).

Table 9 Enzyme activities in transformants carrying all 3 *ara* genes

Strain	L-arabinose isomerase (*araA*) Sp.act umol/min/mg				L-ribulokinase (*araB*) Sp.act umol/min/mg			L-ribulose 5-P 4-epimerase (*araD*) Sp.act umol/min/mg		
	Oct 20-21 (1)	Oct 20-21 (2)	Dec 20-22 (2)		Oct 20-21	Dec 20-22		Oct 20-21	Dec 20-22	
BFY012	nd		nd		nd	nd		nd	nd	
BFY013	0.05	0.10	0.11	trp	1.2	1.3	ura	0.5	1.0	his
BFY014	0.04	0.11	0.11	trp	1.2	1.9	his	nd	nd	ura
BFY015	nd		nd	ura	2.4	2.8	trp	0.39	0.9	his
BFY016	0.03		0.06	his	2.4	2.4	trp	nd	nd	ura
BFY017	0.02		nd	his	0.9	1.1	ura	1.56	>3.5	trp
BFY018	0.01		nd	ura	1.2	1.0	his	1.12	>2.2	trp
Zymomonas	0.85				0.9			1.9		

nd=not detected
(1)=cysteine-carbazole
(2)=new method, NADH disappearance
All cultures were grown in selective medium (YNB+2%gal-trp-his-ura)

Table 10 Enzyme Activities in Strains Used for Fermentation

Strain	MnCl2 (20 mM)	Protein mg/ml	Isomerase (*araA*) Sp.act umol/min/mg	Kinase (*araB*) Sp.act umol/min/mg	Epimerase (*araD*) Sp.act umol/min/mg
BFY012	No	6.6	nd	nd	nd
BFY012	Yes	6.1	nd	nd	nd
BFY013	No	7.0	0.09	2.2	0.6
BFY013	Yes	6.7	0.09	1.9	0.5

Example 14—Whole-cell Fermentation in *S. cerevisiae*

[0146] The transformed yeast cells carrying bacterial genes *araBAD* were first grown in galactose or arabinose alone or in the presence of both galactose and arabinose in YNB. Cells were collected by centrifugation and washed in water. The washed cells were resuspended in liquid media containing 1% yeast extract and 2% peptone (YP). The cell suspensions were aliquoted into various tubes before appropriate sugars were added. The tubes were incubated at 30°C and cell samples were taken at the time indicated. The samples were filtered and analyzed for ethanol concentration by gas chromatography (GC) according to Tietz, 1976 (Table 11 and Fig. 15) or by high perfoemanc liquid chromatography (HPLC). As shown in Table 11, cells that were grown in both galactose and arabinose immediately

before the fermentation assay had a slightly higher overall yield of ethanol than cells grown in galactose alone during the same period. The strains BFY534 and BFY535 were grown in arabinose alone prior to fermentation. From a starting concentration of 19 g/L of L-arabinose, BFY534 and BFY535 used 12.7 and 11.8 g/L of L-arabinose to yield 4.7 and 4.9 g/L of ethanol in 48 hours respectively. The percentage of maximum theoretical conversion would thus be 75% and 78% respectively and a productivity of 0.012 g EtOH/g cells hr for both strains. In an additional fermentation with strain BFY534 performed in shake flasks, 19.7 g/L of L-arabinose was converted to 8.5 g/L ethanol in 96 hrs giving 85% of the theoretical maximum conversion and a productivity of 0.017 g EtOH/g cells hr.

Table 11 Ethanol Concentration (g/l) from Whole Cell Fermentation

	Glucose (66.7g/l)		Arabinose (66.7g/l)			No sugar		
Time (hrs)	0	24	0	24	120	0	24	120
BFY012 (Gal)	1.9	33.6	1.0	1.1	1.8	1.0	0.9	1.8
BFY013 (Gal)	1.6	33.3	0.9	1.3	2.6	0.0	0.9	0
BFY015 (Gal)	1.5	33.8	0.9	1.3	2.5	0.0	0.9	1.3
BFY012 (G+A)	2.1	34.3	1.0	1.2	1.9	1.0	1.0	1
BFY013 (G+A)	1.7	34.2	1.0	1.8	4.2	0.9	1.0	0
BFY015 (G+A)	1.5	33.8	0.9	1.5	3.2	0.0	1.0	0

Example 15—Cell-free Fermentation in *S. cerevisiae*

[0147] For fermentation of arabinose in a cell-free system, yeast transformants were grown in the presence of both galactose and arabinose in YNB. Cells were collected by centrifugation and washed in water. Cell walls were removed by enzymatic digestion and the cells were then lysed in a lysis buffer containing 20mM potassium phosphate buffer, pH 7 and 10mM $MgCl_2$ and 1mM DTT. Cells debris were removed by centrifugation, and the supernatant was transferred to a tube where various chemicals were added to the supernatant such that the fermentation mix contained 7mM Mg acetate, 5mM ATP, 0.1 mM diphosphoglyceric acid, 4mM Na arsenate and 2mM NAD^+. Fermentation was started by adding appropriate sugar to the fermentation mix in the tube. The tube was incubated at 30° C, and samples were taken at the time indicated. The samples were boiled, centrifuged, filtered and analyzed for ethanol concentration by gas chromatography (GC) as previously described. Results of the cell-free fermentation are shown in Table 12, Fig. 16, Fig. 17 and Fig. 18.

Table 12 Ethanol Concentration (g/l) from Cell-Free Fermentation

	Glucose					Arabinose					No sugar				
Time (hrs)	0	2	24	48	72	0	2	24	48	72	0	2	24	48	72
BFY012	0.0	2.7	8.1	8.3	8.2	0.0	1.4	1.9	1.9	1.9	1.2	1.5	1.9	1.9	2.0
BFY013	0.0	3.1	12.6	13.2	12.8	0.0	1.7	4.5	6.0	6.8	0.0	1.5	2.1	2.1	2.0
BFY012 (20 mM $MnCl_2$)	1.3	2.5	9.2	9.0	8.5	1.2	1.4	1.9	1.9	2.1	0.0	1.4	2.0	2.0	1.8
BFY013 (20 mM $MnCl_2$)	1.3	3.0	12.8	13.1	-	1.3	1.8	4.6	6.1	6.8	1.3	1.6	2.0	2.0	-

Example 16-Mixed-Sugar Fermentation in S. cerevisiae

[0148] Yeast strains adapted to contain the *araB* and *araD* genes from *E. coli*, the *araA* isomerase gene from *B. subtilis*, and the *GAL2* overexpression plasmid have been described previously (see for example, Example 11). Adapted strains, BFY534 for example, were tested for fermentation of glucose, arabinose, and a mixture of arabinose and glucose. In particular, 125 ml non-baffled flasks containing 50 ml of yeast-extract-peptone media (including adapted yeast) and either no sugar, glucose, L-arabinose, or both glucose and L-arabinose were prepared. The flasks were closed with Saranwrap held in place with rubber bands. The fermentations were performed at 30°C with gentle shaking (80rpm). In all cases, each sugar was present at a concentration of 20 g/L.

[0149] The results are illustrated in Fig. 19, where a greater than 50% increase in ethanol production was obtained in co-fermentation of glucose and L-arabinose, compared to the ethanol yield of glucose alone.

[0150] While a number of exemplary aspects and embodiments have been discussed above, those of skill in the art will recognize certain modifications, permutations, additions and sub-combinations thereof. It is therefore intended that the following appended claims and claims hereafter introduced are interpreted to include all such modifications, permutations, additions and sub-combinations as are within their true spirit and scope

[0151] This specification contains numerous citations to references such as patents, patent applications, and scientific publications. Each is hereby incorporated by reference for all purposes.

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CLAIMS

We claim:

1. A method for producing an L-arabinose utilizing yeast strain for the production of ethanol, comprising the steps of:

modifying the yeast strain by introducing genes for the expression of an *araA* peptide, an *araB* peptide and an *araD* peptide to produce a transgenic yeast strain having the capacity to consume L-arabinose, and to produce ethanol from a material comprising L-arabinose.

2. The method according to claim 1, wherein the *araA* gene is derived from *E. coli* or *B. subtilis*.

3. The method of claim 1 wherein the yeast strain has at least one genomic mutation in the aldose reductase (AR) gene that renders the aldose reductase inactive.

4. The method of claim 1 wherein the yeast strain having a genomic mutation in the AR gene is obtained by screening for spontaneous mutations.

5. A yeast strain produced according the method of claim 1.

6. The method of claim 1, further comprising steps of:

providing an arabinose-containing material; and

performing simultaneous saccharification and fermentation of the arabinose-containing material by use of the transgenic yeast strain.

7. The method of claim 1, wherein the step of modifying the yeast strain includes transforming the yeast strain for the expression of at least one arabinose transporter encoded by a gene selected from the group consisting of *GAL2*, *KmLAT1* and *PgLAT2*.

8. An ethanol producing yeast strain comprising at least one heterologous arabinose transporter wherein the ethanol producing yeast strain is aldose reductase (AR) deficient (AR$^-$).

9. The ethanol producing yeast strain of claim 8 further comprising at least two heterologous arabinose transporters.

10. The ethanol producing yeast strain of claim 8 wherein the heterologous arabinose transporter is from a non-conventional yeast species.

11. The ethanol producing yeast strain of claim 10 wherein the heterologous arabinose transporter is selected from KmLat1 or PgLat2.

12. The ethanol producing yeast strain of claim 8 further comprising overexpression of a conventional yeast derived *GAL2*-encoded galactose permease.

13. The ethanol producing yeast strain of claim 12 wherein the yeast derived *GAL2*-encoded galactose permease is under control of a *TDH3* promoter.

13. The ethanol producing yeast strain of claim 8 further comprising one or more bacterial genes wherein the one or more bacterial genes facilitate arabinose utilization and fermentation in the ethanol producing yeast strain.

14. The ethanol producing yeast strain of claim 13 wherein the one or more bacterial genes for facilitated arabinose utilization and fermentation are selected from the group consisting of *araA*, *araB* and *araD*.

15. The ethanol producing yeast strain of claim 13 comprising *araA*, *araB* and *araD*.

16. The ethanol producing yeast strain of claim 15 wherein at least one of *araA*, *araB* and *araD* is derived from *E. Coli*.

17. An arabinose fermenting yeast strain deposited with American Type Culture Collection (ATCC) as Accession Number _____.

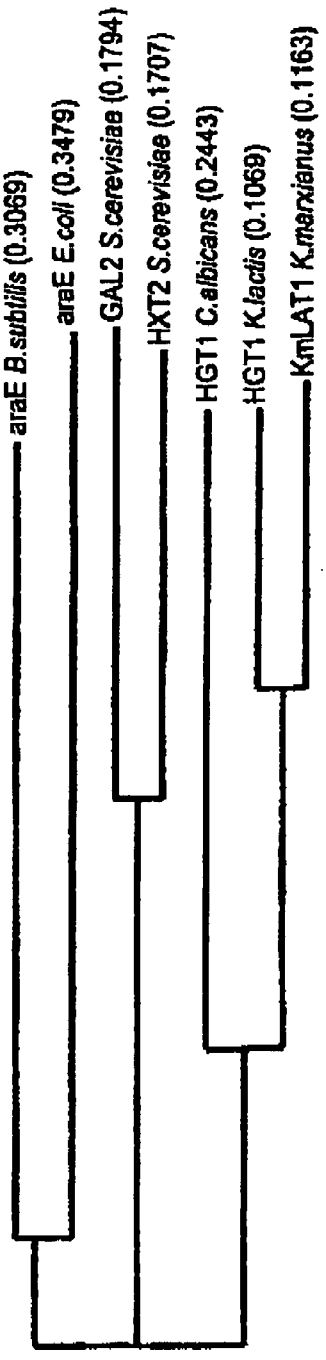
araE B.subtilis (0.3069)
araE E.coli (0.3479)
GAL2 S.cerevisiae (0.1794)
HXT2 S.cerevisiae (0.1707)
HGT1 C.albicans (0.2443)
HGT1 K.lactis (0.1069)
KmLAT1 K.marxianus (0.1163)

Fig. 1

Fig. 2A

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    H T L K  D K L  L L R  N I E F  K G T  F Y A  K F P Q  I H N  I Y A  I G V   33 SEQ ID NO 2
1   ATGACTTTAA AAGATAAACT ATTGCTCCGC AATATCGAAT TCAAGGGAAC TTTCTATGCG AAGTTCCCGC AAATTCACAA CATTTACGCA ATCGGTGTGA    SEQ ID NO 1

    I S C I  S G L  H F G F  D I S  S H S  S H I G  T C T  Y K K  Y F D H  P K S   67
101 ATTCGTGTAT ATCTGGTCTC ATGTTTGGTT TCGATATCTC TTCAATGTCT TCCATGATCG GTACTGAAAC TTACAAAAAA TATTTTGACC ATCCAAAATC

    I T Q  G G I  T A S  H S G  G S F  L G S  L L S  P A I  S D T  F G R  K V S   100
201 CATTACCCAA GGTGGTATCA CCGCGTCAAT GTCCGGTGGT TCCTTCTTAG GCTCTTTACT CTCTCCTGCT ATTTCCGATA CCTTTGGCAG AAAAGTGTCG

    L H I C  A V L  N I V  G C I  L Q S  A Q D  Q P H  L I A  G R V  I A G  L   133
301 TTGCACATTT GTGCCGTCTT GTGGATCGTC GGATGCATTT TGCAAAGTGC TGCCCAAGAC CAACCAATGC TAATCGCTGG CCGTGTTATC GCAGGGTTGG

    G I G  F G S  G S A  P I Y  C S E  I S P  P K V  R G L  I T G  L F Q  F S I T   167
401 GTATCGGGTT CGGCTCTGGT TCTGCTCCAA TTTACTGTTC TGAAATCTCC CCACCAAAGG TTAGAGGCTT GATCACCGGT CTTTTCCAGT TCTCTATCAC

    V G I  M I L  F Y V  G Y G  C H F  L S G  N L S  F R L  T W G  L Q V  I P G   200
501 TGTTGGTATT ATGATTCTCT TCTACGTTGG TTACGGGTGC CACTTCCTCA GTGGTAATCT TTCATTCAGA TTGACTTGGG GTTTGCAAGT TATCCCAGGA

    F V L  L V G  V L F  L P E  S P R  W L A  N H D  R W E  E T E  S I V  N K V   233
601 TTTGTGTTGC TGGTCGGTGT CCTATTCTTG CCAGAATCCC CACGTTGGTT GGCTAACCAC GACCGTTGGG AAGAAACTGA GTCAATCGTC GCCAAGGTCG

    V A K  G N V  D D E  E V K  F Q L  E E I  N E Q  V I L  D A A  A K N  F S F  K   267
701 TCGCCAAGGG TAACGTAGAC GATGAAGAAG TCAAGTTCCA ATTGGAAGAA ATTAAACAGC AGGTGATTCT TGATGCTGCC GCCAAGAACT TCTCCTTCAA

    D L L  R P R  T R K  K L F  V G V  C A Q  H W Q  Q L C  G H N  V H N  Y Y I   300
801 GGATTTGCTA AGACCAAAGA CCAGAAAGAA GCTCTTTGTT GGTGTGTGTG CTCAAATGTG GCAACAATTG TGTGGTATGA ACGTTATGAT GTACTACATT
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Fig. 2B

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                V  Y  V  F  N  N  A  G  Y  Y  T  G  N  T  N  L  V  A  S  S  I  Q  Y  V  L  N  V  L  N  T  F  P  A  L   333
901  GTGTACGTCT TTAACATGGC TGGTTACACT GGTAACACCA ACTTGGTTGC AICTTCGATT CAATAGTCT TGAACGTGCT AATGACTTTC CCTGCACTAT

                F  L  I  D  K  V  G  R  R  P  V  L  I  V  G  G  I  F  M  F  T  W  L  F  A  V  A  G  L  L  A  S  Y  S   367
1001 TCTTAATCGA TAAAGTCGGT AGAAGACCTG TCTTGATCGT TGGTGGTATT TTCATGTTCA CATGGTTGTT CGCTGTGGCT GGTTTGTTGG CATCATATTC

                V  P  A  P  N  G  V  N  G  D  D  T  V  T  I  R  I  P  D  R  K  K  S  A  A  K  G  V  I  A  C  S  Y   400
1101 CGTCCCAGCT CCAAATGGTG TTAACGGTGA TGATACTGTC ACAATCAGAA TCCCAGACAA GCACAAGTCC GCGCTAAGG GTGTCATTGC ATGTTCATAC

                L  F  V  C  S  F  A  P  T  K  G  I  G  I  W  I  Y  C  S  E  I  F  N  N  M  E  R  A  K  G  S  S  V   433
1201 TTGTTCGTCT GCTCTTTCGC TCCAACTGG GGTATTGGTA TCTGAATTTA CTGTTCCGAA ATTTTCAACA ACATGGAAAG AGCCAAGGGT TCCTCTGTGG

                A  A  A  T  N  M  A  F  N  F  A  L  A  M  E  V  P  S  A  F  K  N  I  S  W  K  T  Y  I  V  F  G  V  F   467
1301 CTGCTGCTAC CAACTGGTGA TTCAACTTCG CTTTGGCGAT GTTCGTCCCA TCTGAATTCA AGAACATCTC ATGGAAACCA TACATCGTCT TTGGTGTCTT

                S  V  A  L  T  V  Q  T  Y  F  M  F  P  E  T  R  G  K  T  L  E  E  I  D  Q  N  W  V  D  N  I  P  A   500
1401 TTCAGTTGCA TTGACTGTCC AAACTACTT CATGTTCCCA GAAACTAGAG GTAAGAACCTT GGAAGAATC GACCAATGT GGTCGACAA CATCCCAGCC

                W  K  T  S  S  Y  I  P  Q  L  P  I  I  E  D  E  F  G  N  K  L  G  L  L  G  N  P  Q  H  L  E  H  V   533
1501 TGGAAGACTA CCAGCTACAT CCCACAATTG CCTATCATCG AAGATGAATT TGGTAACAAG TTGGGTTTGT TGGGTAACCC ACAACATCTC GAGCATGTTA

                K  S  V  E  K  D  T  V  V  E  K  L  E  S  S  E  A  N  S  S  S  S  V  *   556
1601 AATCCGTCGA AAAGGATACT GTAGTGGAAA AATTAGAATC GTCAGAGGCT AATAGCAGCA GCTCGGTCTA G
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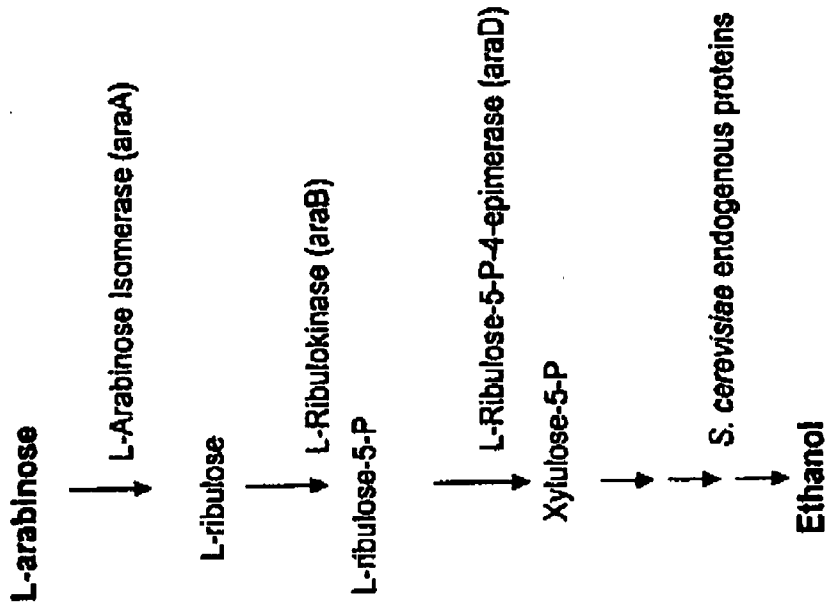
L-arabinose
L-Arabinose Isomerase (araA)
L-ribulose
L-Ribulokinase (araB)
L-ribulose-5-P
L-Ribulose-5-P-4-epimerase (araD)
Xylulose-5-P
S. cerevisiae endogenous proteins
Ethanol

Fig. 3

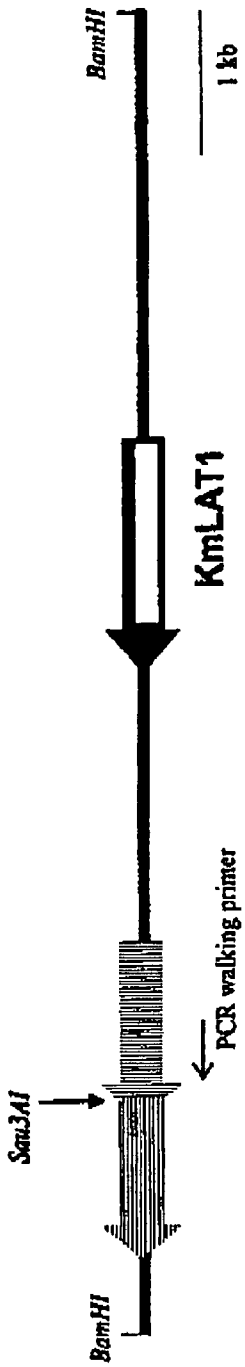

Fig. 4

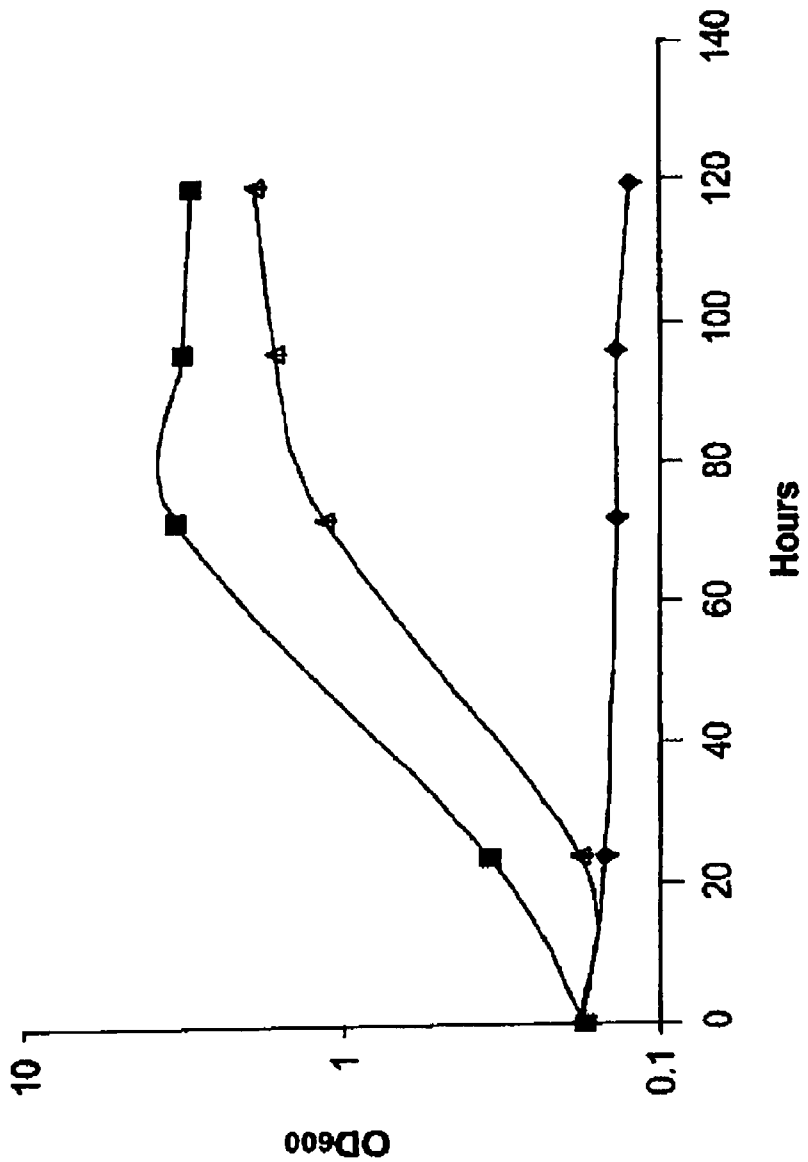

Fig. 5

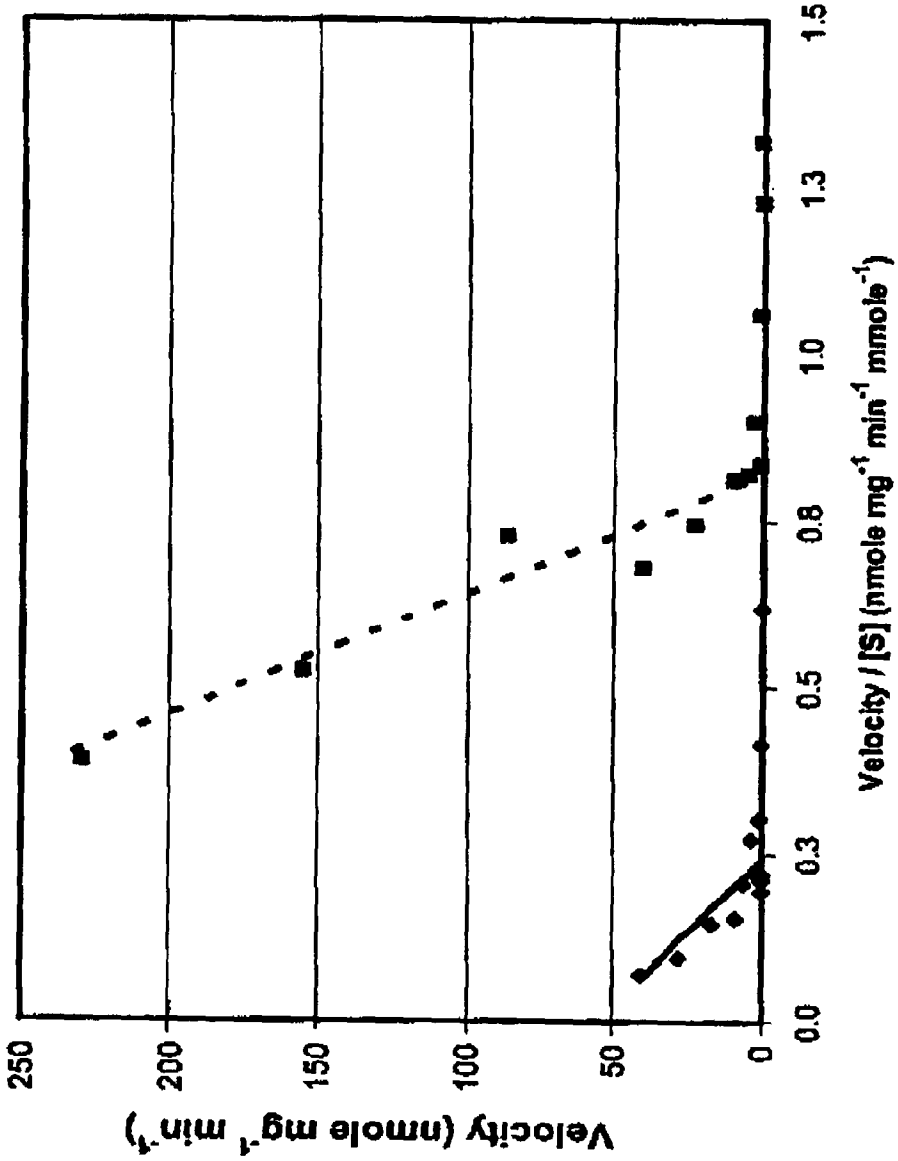
Velocity (nmole mg-1 min-1)
250
200
150
100
50
0
0.0
0.3
0.5
0.8
1.0
1.3
1.5
Velocity / [S] (nmole mg-1 min-1 mmole-1)

Fig. 6A

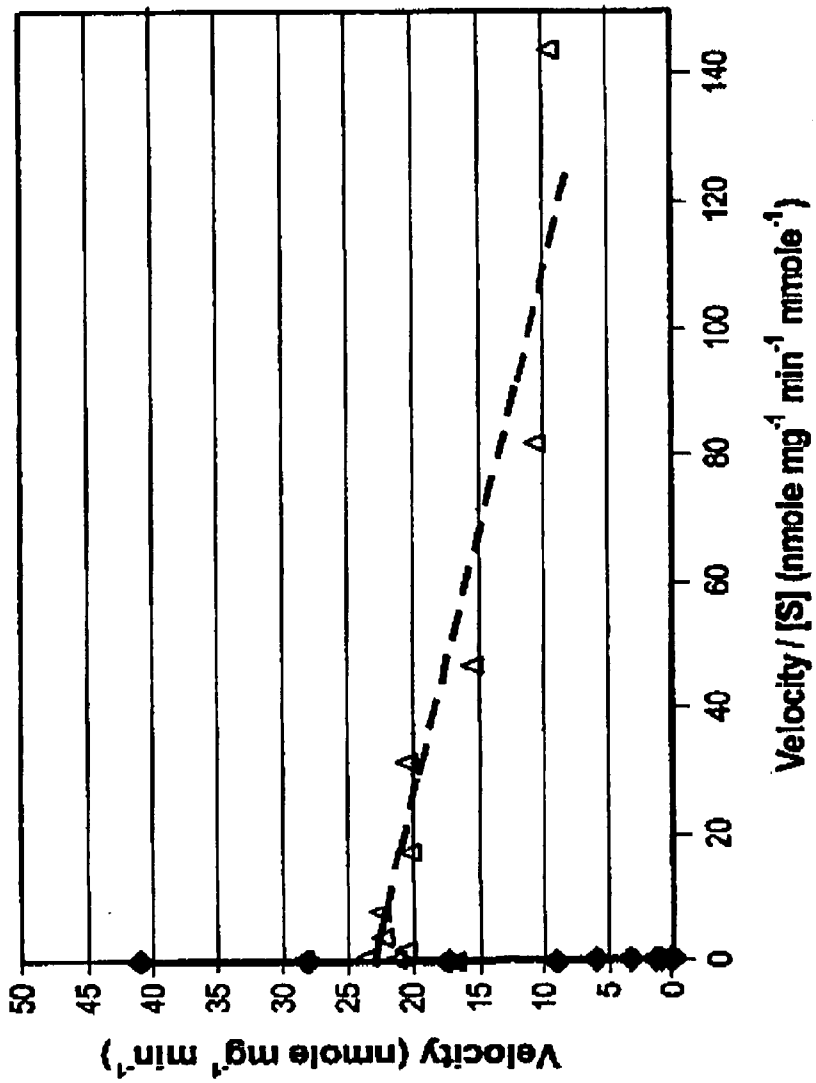
Velocity (nmole mg^{-1} min^{-1})
50
45
40
35
30
25
20
15
10
5
0
Velocity / [S] (nmole mg^{-1} min^{-1} nmole^{-1})
0
20
40
60
80
100
120
140

Fig. 6B

Fig. 7A

```
SEQ ID No4: 1  M A Y E D K L V A P A L K F R N F L D K
SEQ ID No3: 1  ATGGCTTACGAGGACAAACTAGTGGCTCCGGCCTTGAAGTTTAGAAACTTTCTTGACAAA

           21  T P N I Y N P Y I I S I I S C I A G M M
           61  ACTCCCAATATCTACAATCCATATATCATTTCTATAATCTCGTGCATTGCGGGTATGATG
           41  F G F D I S S M S A F V S L P A Y V N Y
          121  TTCGGTTTTGATATTTCTTCAATGTCAGCGTTTGTCAGTTTACCAGCATACGTGAATTAT
           61  F D T P S A V I Q G F I T S A M A L G S
          181  TTCGATACACCTTCAGCAGTGATTCAAGGATTATCACATCTGCCATGGCTTGGGTTCA
           81  F F G S I A S A F V S E P F G R R A S L
          241  TTTTTCGGGTCAATTGCTTCTGCGTTTGTGTCTGAGCCATTTGGAAGACGAGCTTCCTTA
          101  L T C S W F W M I G A A I Q A S S Q N R
          301  CTAACTTGTTCGTGGTTTTGGATGATAGGAGCAGCCATCCAAGCGTCTTCGCAGAACCGA
          121  A Q L I I G R I I S G F G V G F G S S V
          361  GCTCAATTGATTATGGTCGGATTATATCTGGATTTGGGGTTGGTTTCGGGTCGTCTGTG
          141  A P V Y G S E M A P R K I R G R I G G I
          421  GCTCCCGTATATGGCTCCGAGATGGCACCTAGAAAAATTAGAGGAAGAATTGGTGGAATT
          161  F Q L S V T L G I M I M F F I S Y G T S
          481  TTTCAATTATCTGTCACCCTCGGTATCATGATTATGTTCTTCATAAGTTACGGAACTTCT
          181  H I K T A A A F R L L A W A L Q I I P G L
          541  CATATTAAGACTGCGGCAGCTTTCAGGTTAGCCTGGGCACTCCAGATCATCCTGGACTC
          201  L M C I G V F F I P E S P R W L A K Q G
          601  CTCATGTGTATTGGTGTCTTCTTTATTCCAGAATCTCCTAGATGGTTGGCCAAACAAGGT
          221  H W D E A E I I V A K I Q A K G D R E N
          661  CACTGGGACGAAGCCGAAATCATTGTAGCCAAAATTCAAGCCAAAGGAGATCGAGAAAAT
          241  P D V L I E I S E I K D Q L M V D E N A
          721  CCCGATGTTTTGATTGAAATTTCGGAAATAAAAGACCAATTGATGGTTGACGAGAATGCC
          261  K A F T Y A D L F S K K Y L P R T I T A
          781  AAAGCCTTTACCTATGCTGACTTGTTTCGAAAAATATCTTCCCAGAACCATCACAGCC
```

Fig. 7B

```
281   M  E  A  Q  I  W  Q  Q  L  T  G  M  N  V  M  M  Y  Y  I  V
841   ATGTTCGCTCAAATCTGGCAACAATTGACAGGAATGAATGTCATGATGTACTATATCGTT
301   Y  I  F  E  M  A  G  Y  G  G  N  G  V  L  V  S  S  T  I  Q
901   TACATTTCGAAATGGCTGGCTACGGTGGAAATGGAGTGTTGGTATCATCGACAATTCAG
321   Y  V  I  F  V  V  V  T  F  V  S  L  F  E  L  D  K  F  G  R
961   TACGTTATCTTTGTCGTTGTTACATTTGTCTCATTATTCTTTTTGGACAAATTTGGAAGA
341   R  K  I  L  L  V  G  A  A  S  M  M  T  W  Q  F  A  V  A  G
1021  AGAAAATTTTACTTGTCGGAGGCAGCTTCCATGATGACCTGGCAGTTTGCAGTGGCAGGG
361   I  L  A  R  Y  S  V  P  Y  D  L  S  D  T  V  K  I  K  I  P
1081  ATCTTGGCCAGGTACTCGGTCCCGTACGATCTCAGCGATACTGTCAAAATTAAAATTCCT
381   D  N  H  K  S  A  A  K  G  V  I  A  C  C  Y  L  F  V  A  S
1141  GACAATCACAAATCGGCTGCAAAAGGTGTCATTGCATGCTGCTATCTTTTCGTAGCATCG
401   F  G  F  S  W  G  V  G  I  W  L  Y  C  S  E  V  W  G  D  S
1201  TTCGGATTTTCCTGGGGAGTTGGTATCTGGTTATACTGCTCTGAAGTCTGGGGAGACTCA
421   Q  S  R  Q  R  G  A  A  V  S  T  A  S  N  W  I  F  N  F  A
1261  CAATCGAGACAGAGAGGAGCCGCTGTGTCAACTGCTTCAAATTGGATTTTCAATTTTGCG
441   L  A  M  F  T  P  S  S  F  K  N  I  T  W  K  T  Y  C  I  Y
1321  CTCGCCATGTTCACACCATCTTCGTTTAAAATATCACCTGGAAGACATACTGTATTTAT
461   A  T  F  C  A  C  M  F  I  H  V  F  F  F  P  E  T  K  G
1381  GCCACTTTCTGCGCATGTATGTTCATCCATGTGTTCTTCTTCCCAGAACCAAGGGG
481   K  R  L  E  E  I  A  Q  I  W  E  E  K  I  P  A  W  K  T  T
1441  AAGCGCTTGGAAGAAATTGCTCAAATTTGGGAAGAAAAATTCCAGCTTGGAAAACCACC
501   N  W  Q  P  H  V  P  L  L  S  D  H  E  L  A  E  K  I  N  A
1501  AACTGGCAACCTCATGTTCCTTTGTTGTCGGACCACGAACTTGCGGAAAAGATCAATGCC
521   E  H  V  E  N  V  N  S  R  E  Q  S  D  D  E  K  S  Q  V  *
1561  GAACATGTGGAGAACGTGAATTCTAGGGAACAATCGGATGACGAGAAGTCGCAGGTATAA
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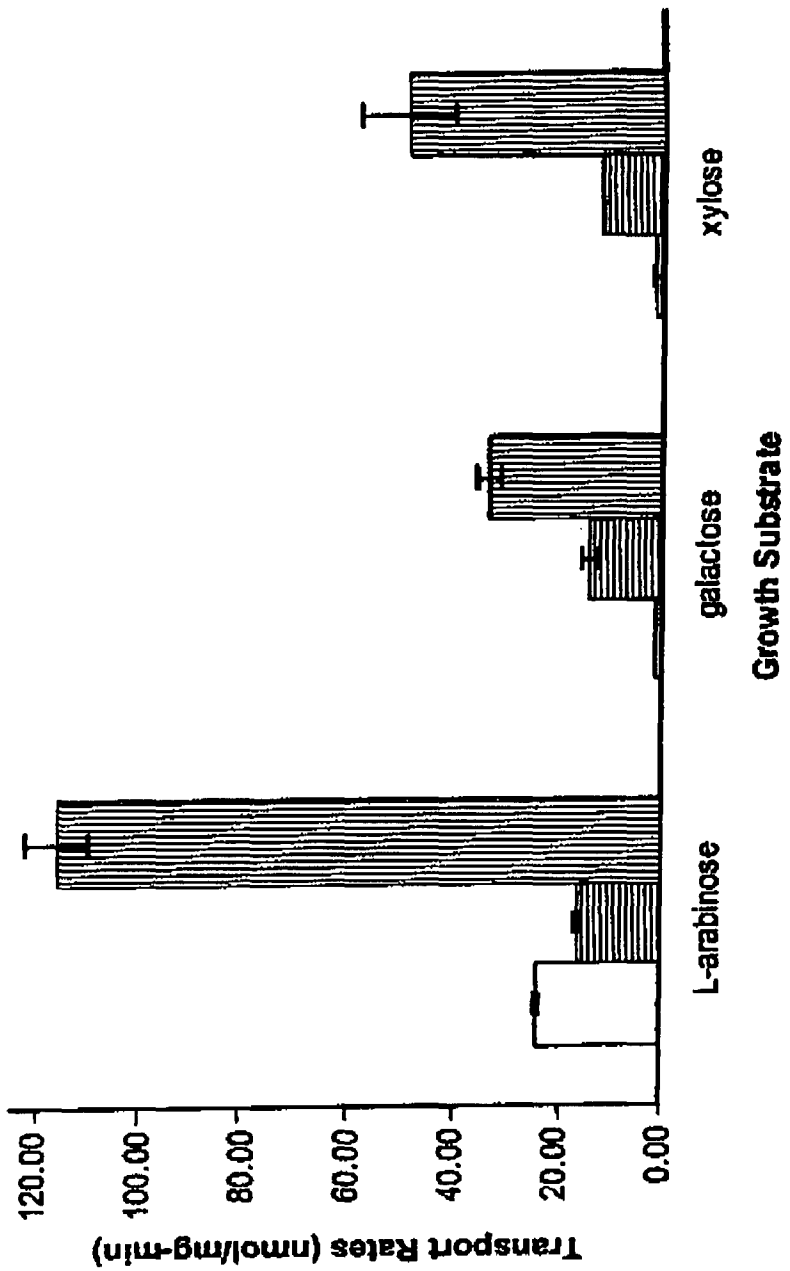
Transport Rates (nmol/mg-min)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
L-arabinose
galactose
xylose
Growth Substrate

Fig.8

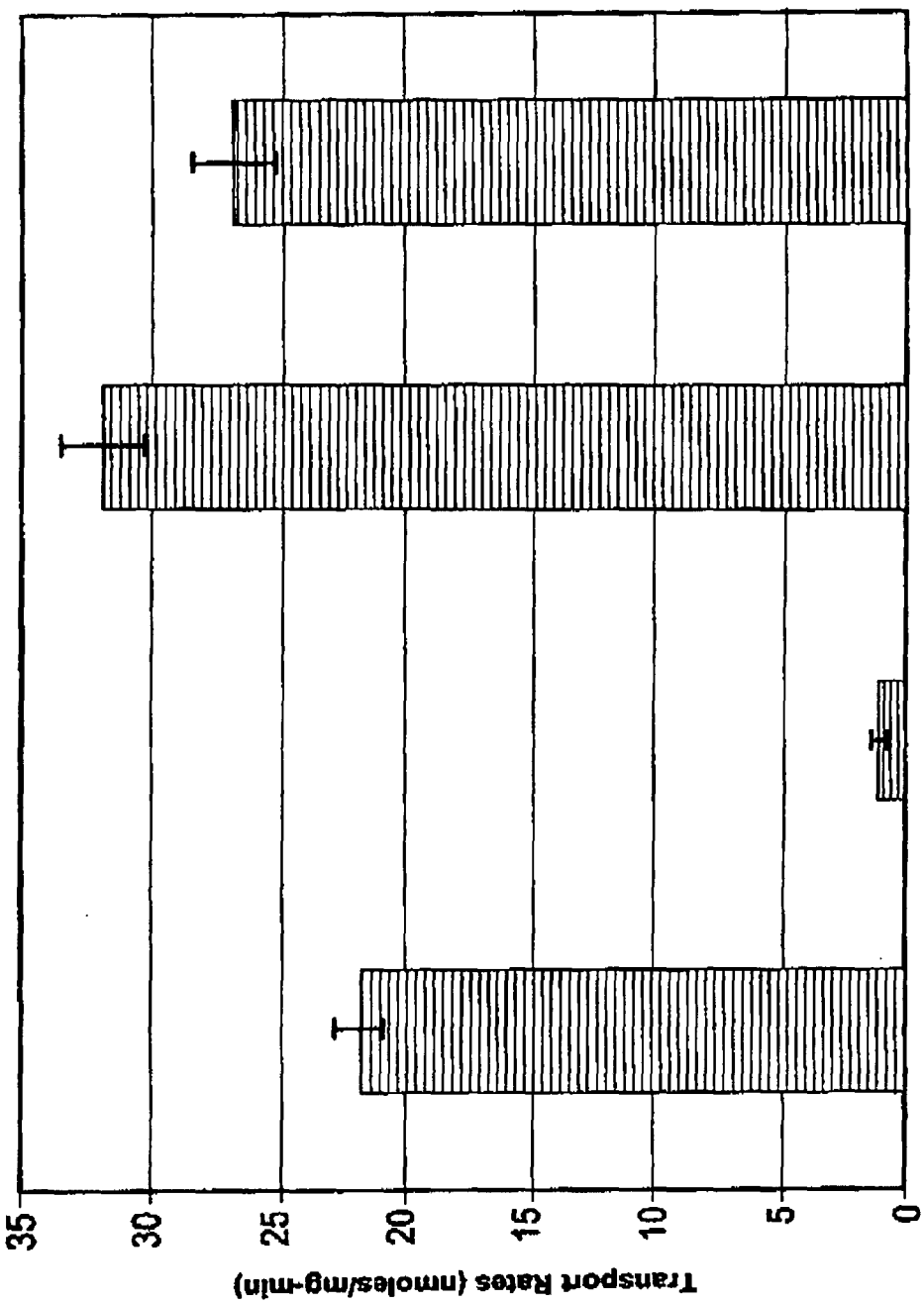
Transport Rates (nmoles/mg-min)
0
5
10
15
20
25
30
35
14C L-arabinose
14C L-arabinose + xylose
14C xylose
14C xylose + L-arabinose
14C-sugar + 100x Competing Sugar

Fig.9

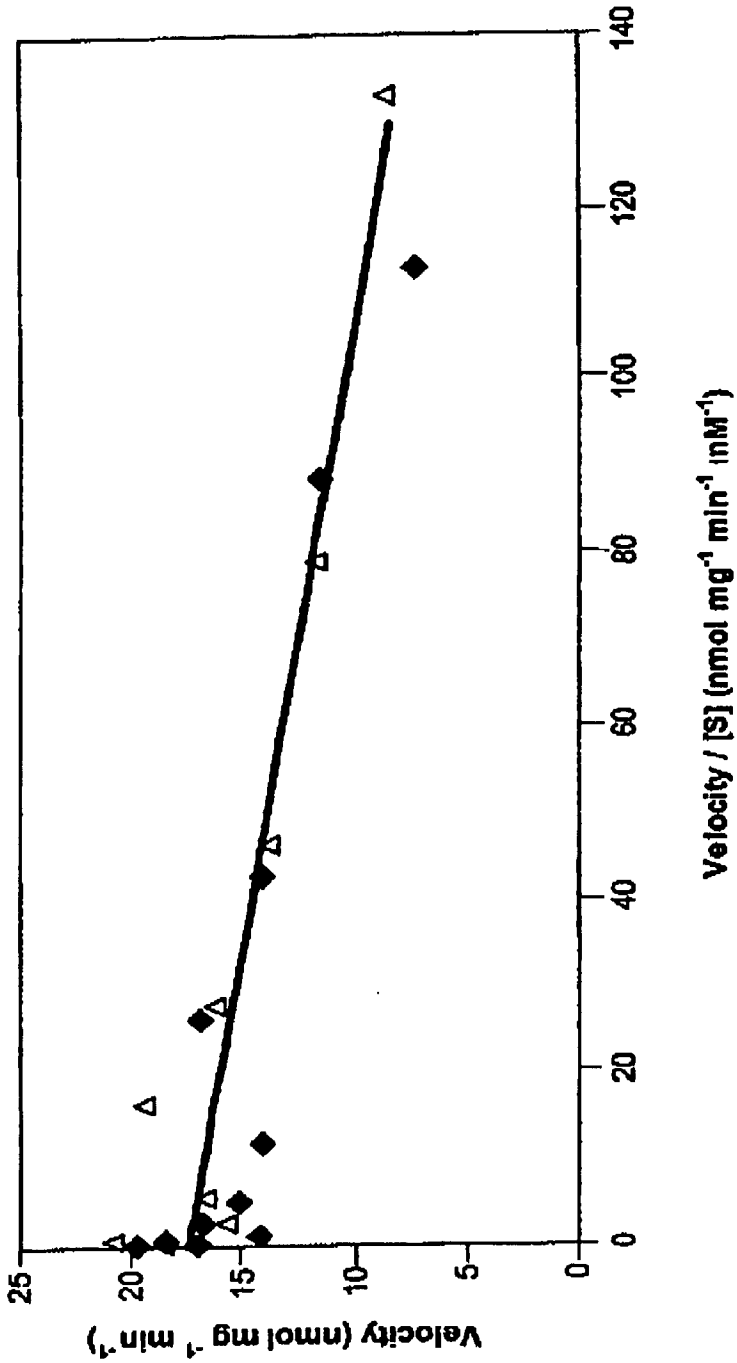
Velocity (nmol mg⁻¹ min⁻¹)
25
20
15
10
5
0
Velocity / [S] (nmol mg⁻¹ min⁻¹ nM⁻¹)
0
20
40
60
80
100
120
140

Fig.10

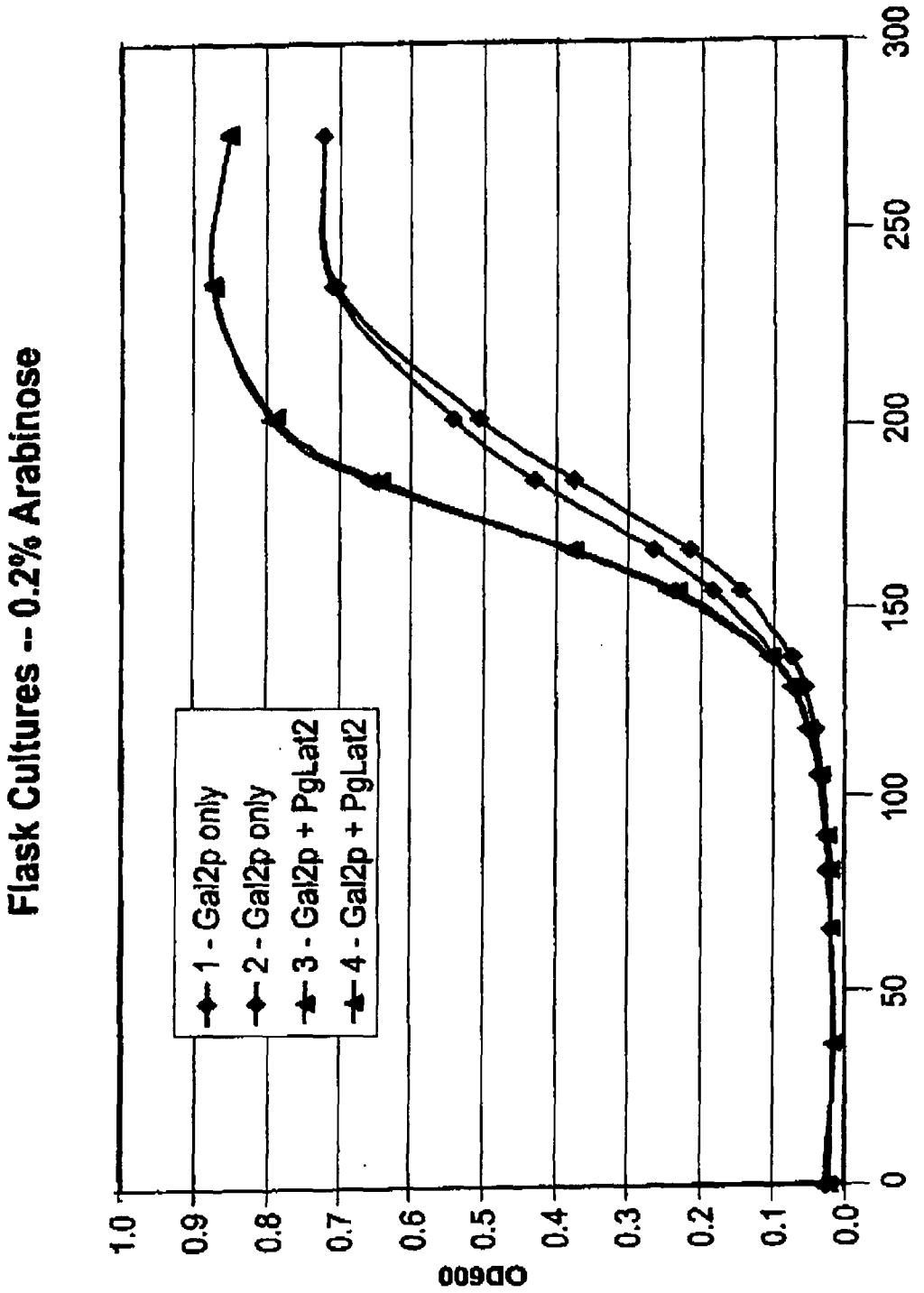
Flask Cultures -- 0.2% Arabinose
1 - Gal2p only
2 - Gal2p only
3 - Gal2p + PgLat2
4 - Gal2p + PgLat2
OD600
Time (hours)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
50
100
150
200
250
300

Fig. 11

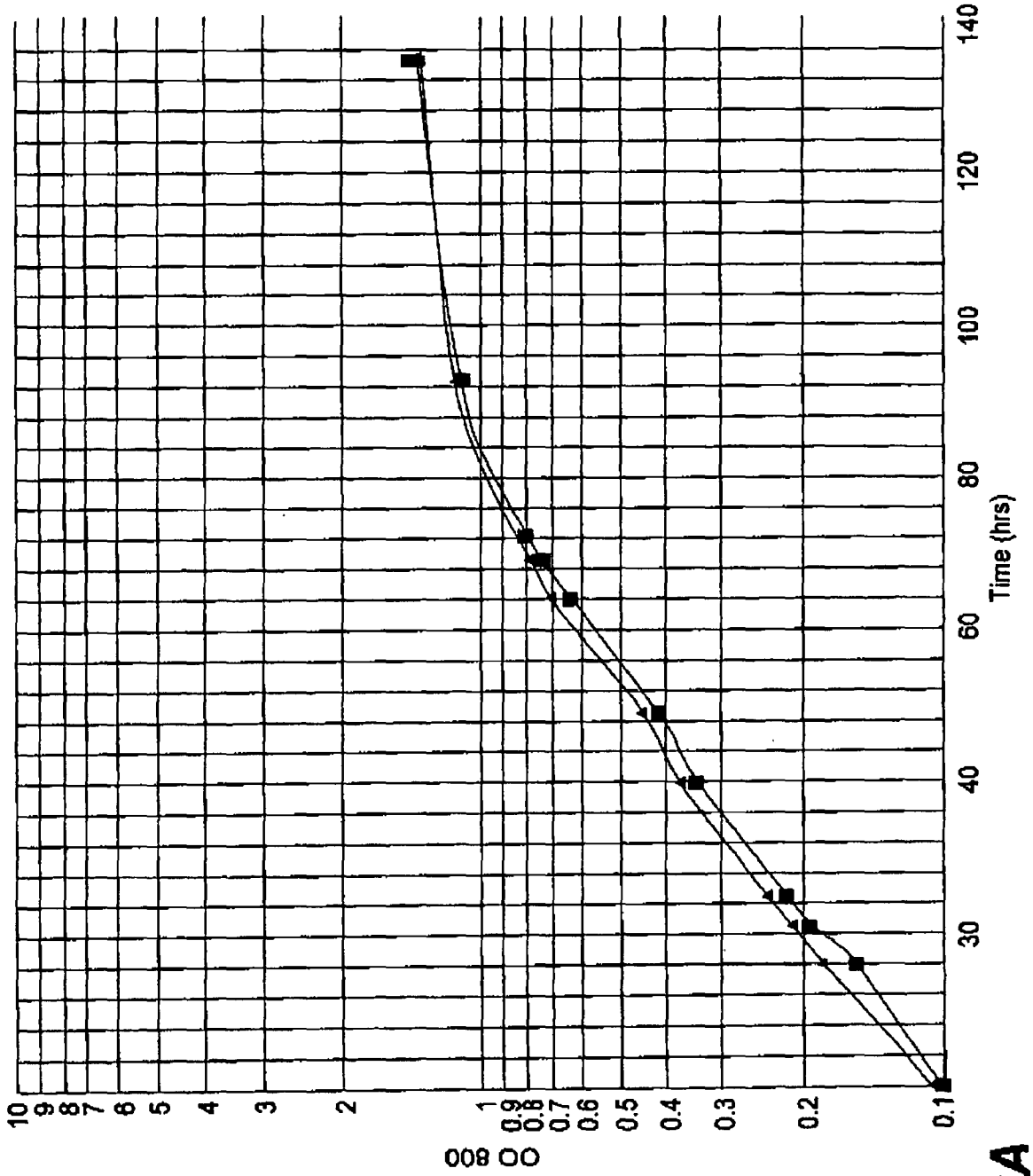
OD 600
10
9
8
7
6
5
4
3
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Time (hrs)
140
120
100
80
60
40
30

Fig. 12A

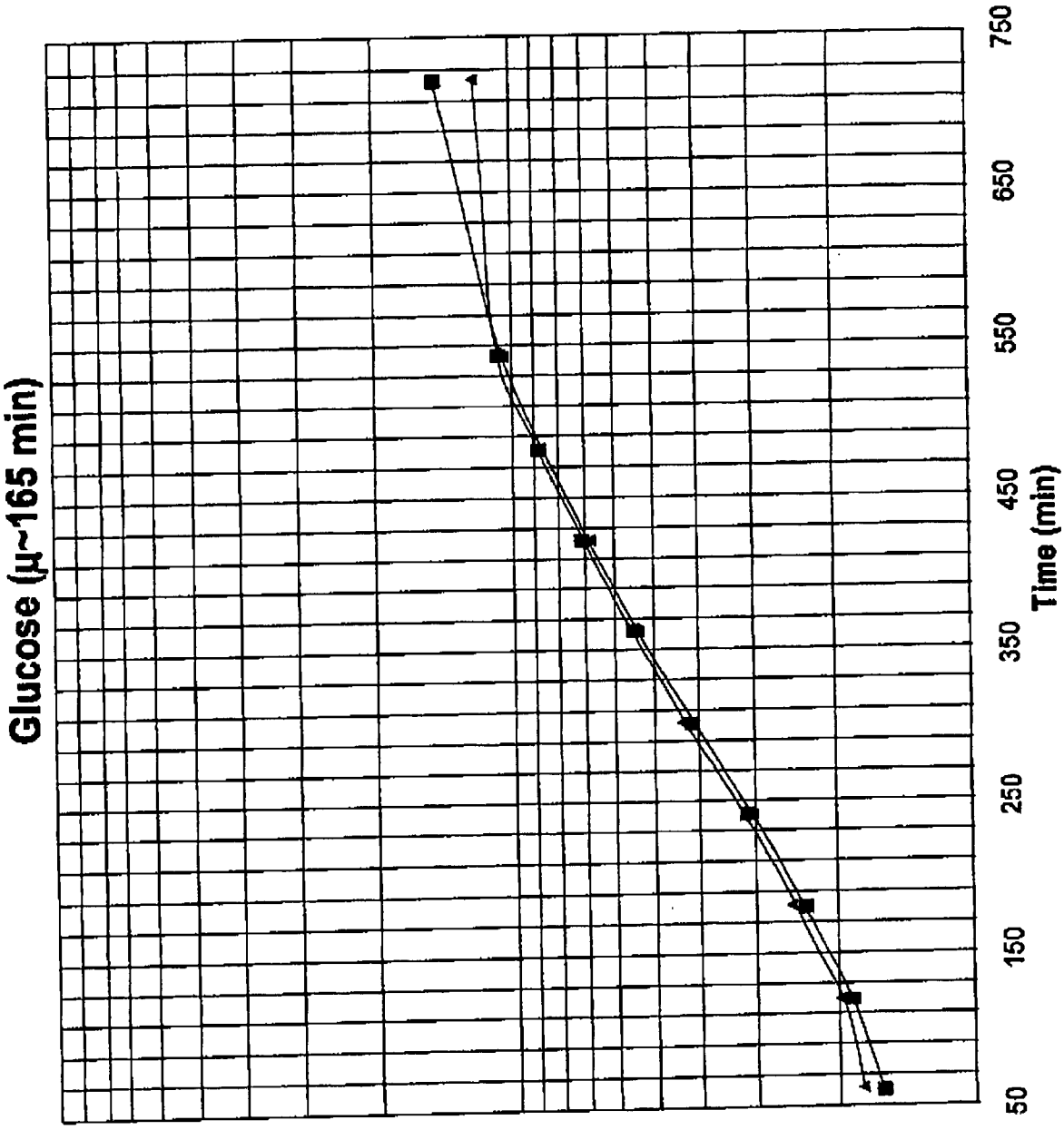

Fig. 12B

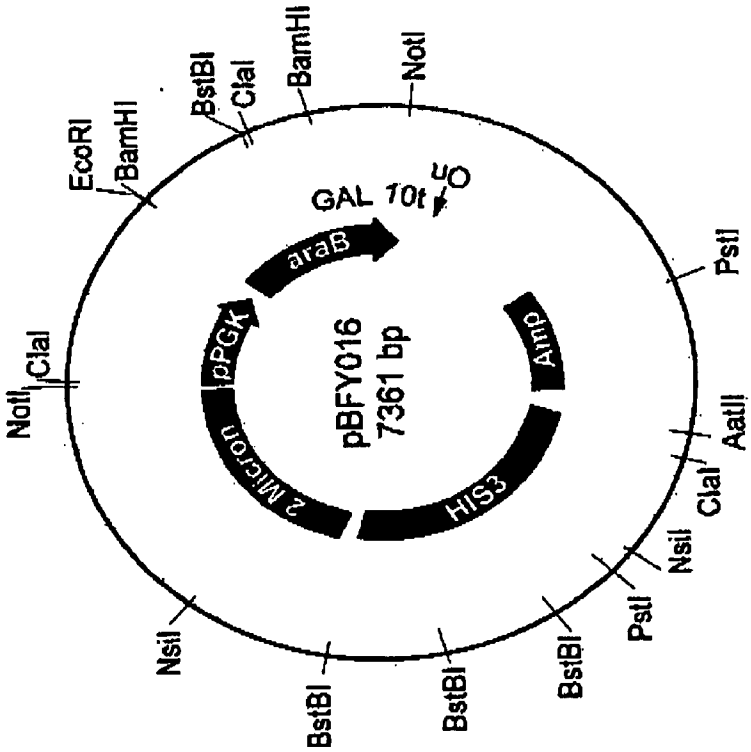

Fig. 13B

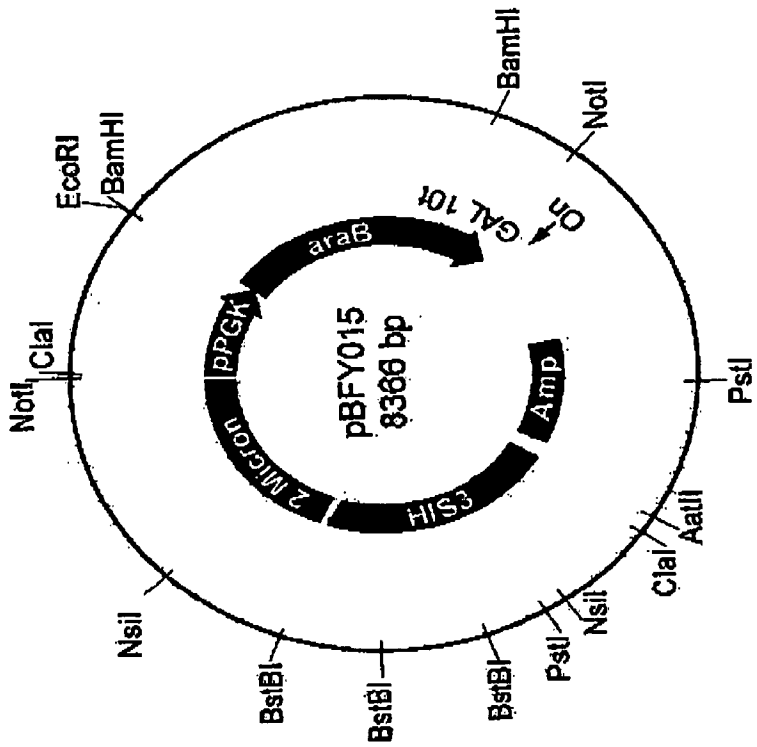

Fig. 13A

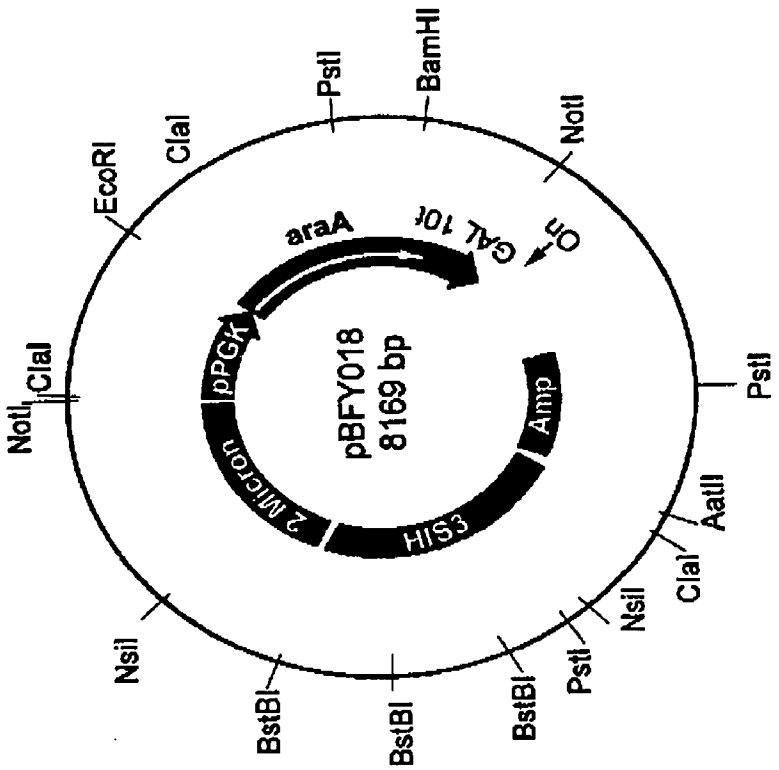
pBFY018
8169 bp
araA
GAL 10t
On
pPGK
2 Micron ori
Amp
HIS3
NotI
ClaI
EcoRI
ClaI
PstI
BamHI
NotI
PstI
AatII
ClaI
NsiI
PstI
BstBI
BstBI
BstBI
NsiI

Fig. 13C

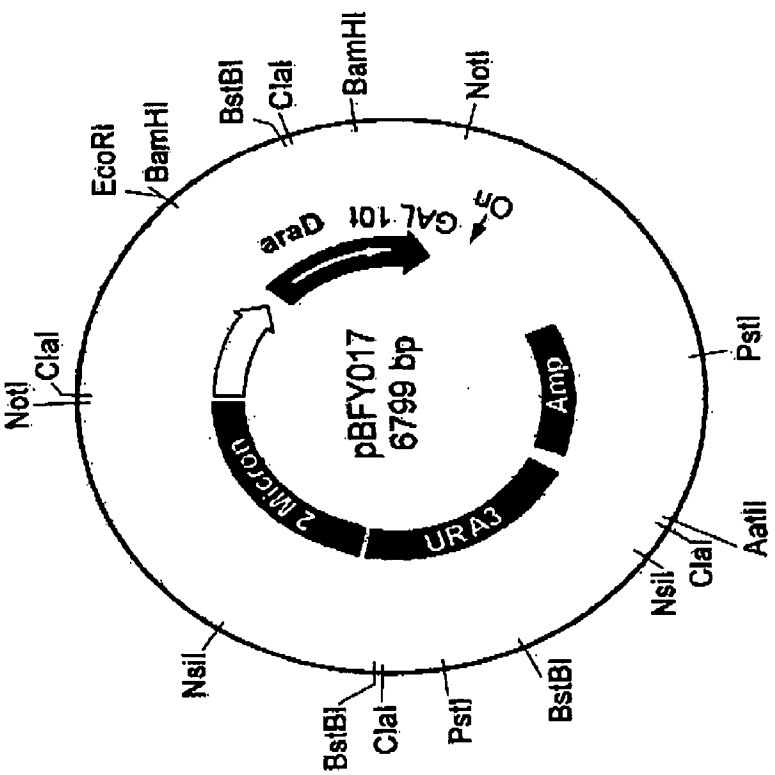

Fig. 14B

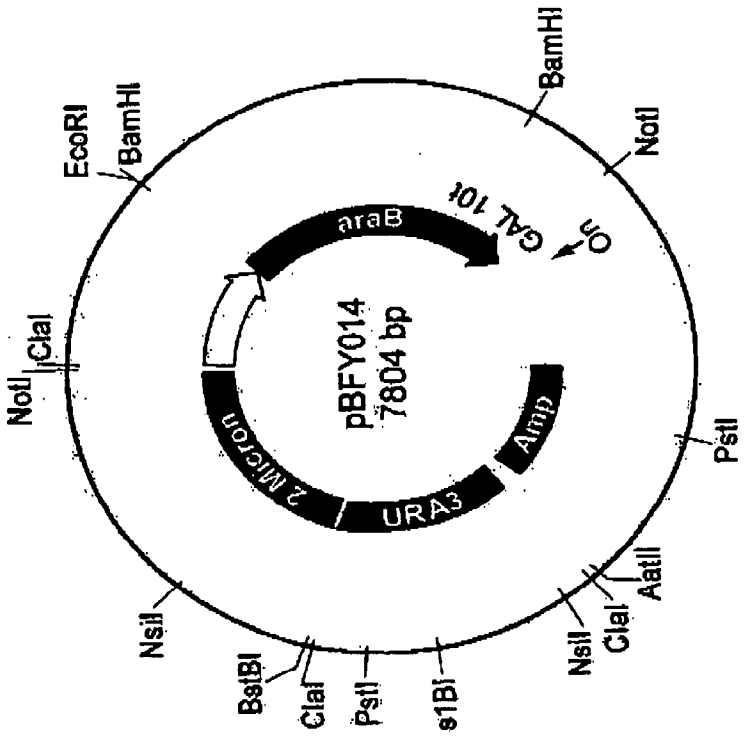

Fig. 14A

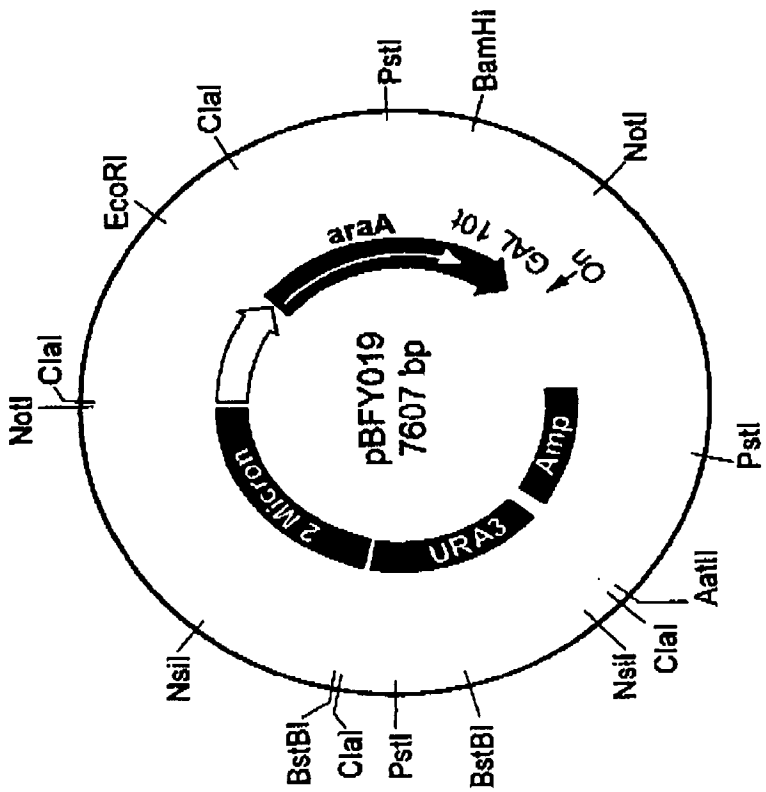
pBFY019
7607 bp
araA
GAL 10t
On
Amp
URA3
2 Micron
NotI
ClaI
EcoRI
ClaI
PstI
BamHI
NotI
PstI
AatII
ClaI
NsiI
NsiI
BstBI
ClaI
PstI
BstBI

Fig. 14C

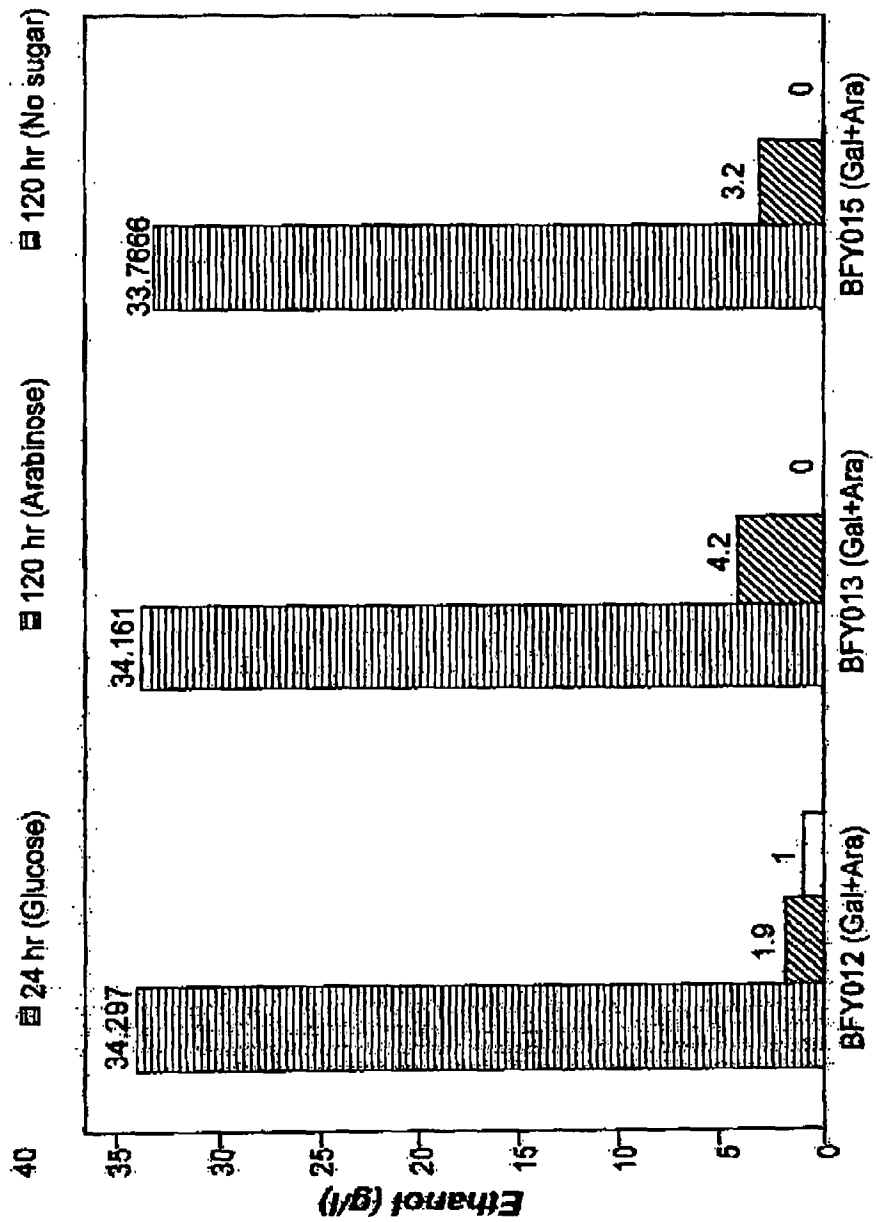
24 hr (Glucose)
120 hr (Arabinose)
120 hr (No sugar)
Ethanol (g/l)
40
35
30
25
20
15
10
5
0
34.297
1.9
1
34.161
4.2
0
33.7666
3.2
0
BFY012 (Gal+Ara)
BFY013 (Gal+Ara)
BFY015 (Gal+Ara)
STRAIN

Fig. 15

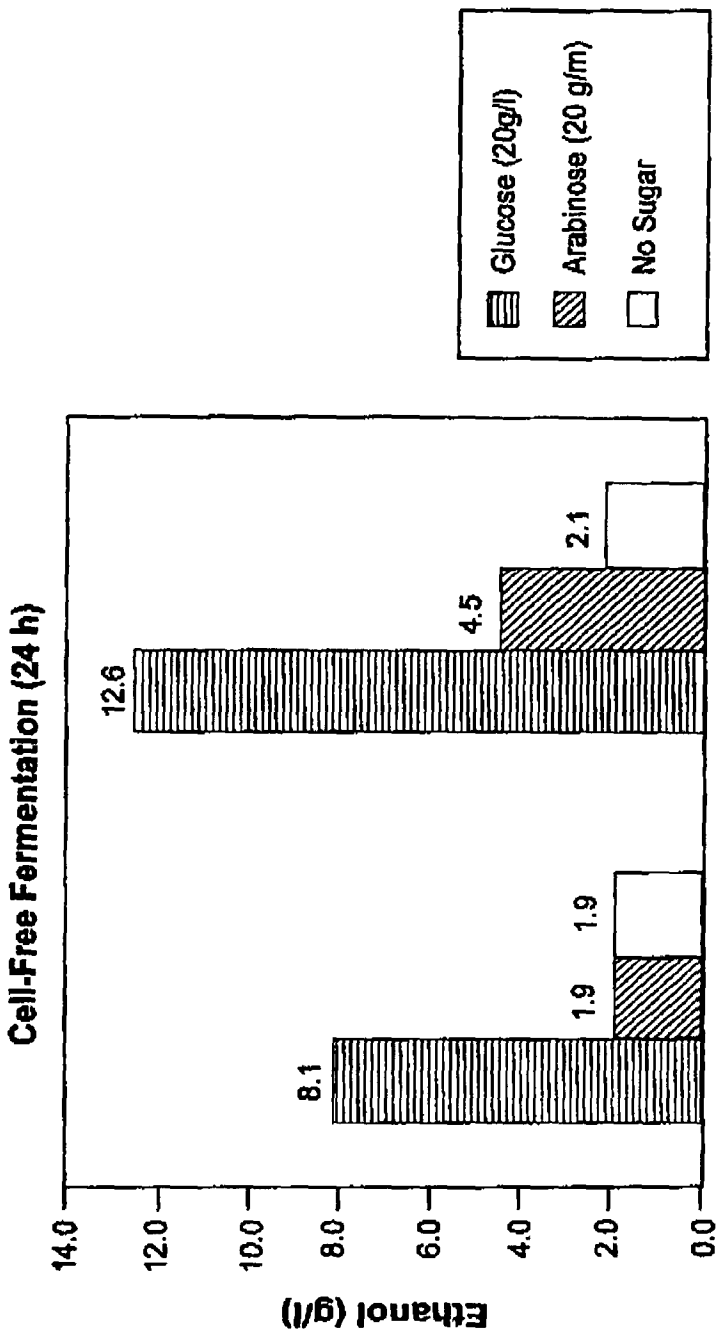
Cell-Free Fermentation (24 h)
Ethanol (g/l)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
12.6
4.5
2.1
8.1
1.9
1.9
BFY013
BFY012
Strains
Glucose (20g/l)
Arabinose (20 g/m)
No Sugar

Fig. 16A

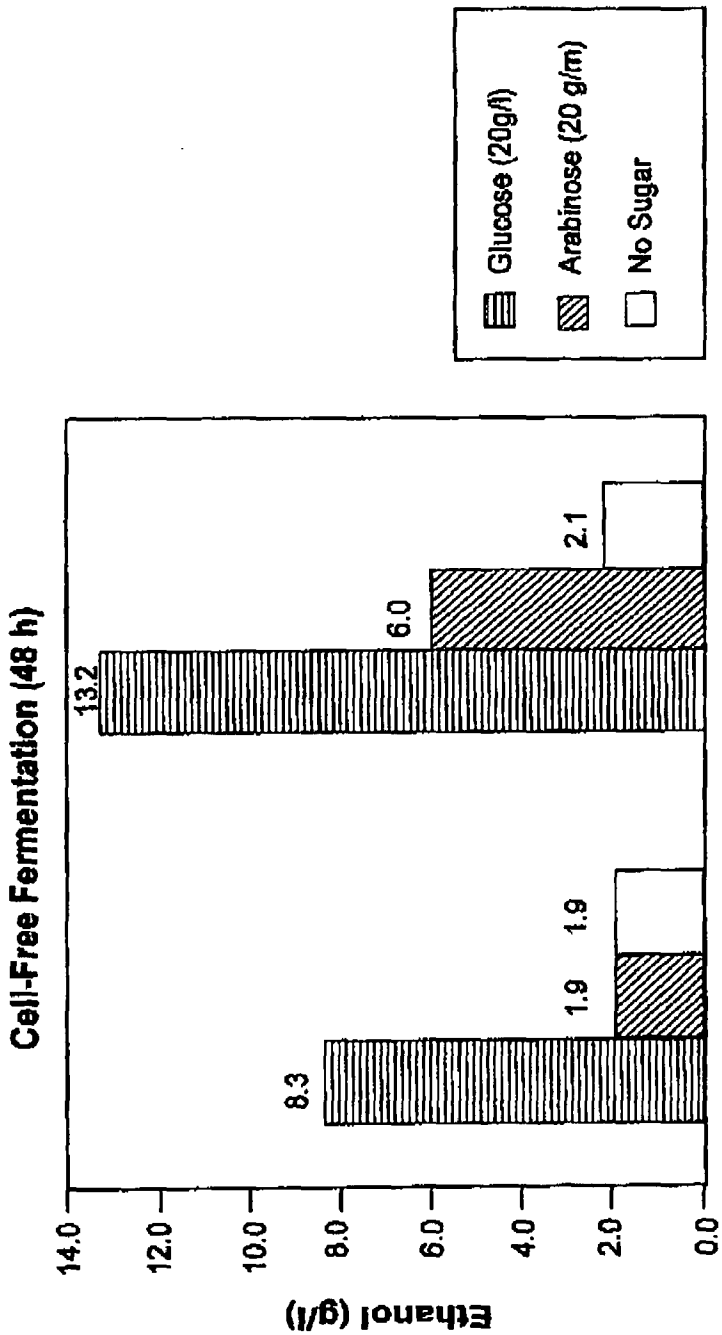
Cell-Free Fermentation (48 h)
Ethanol (g/l)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
13.2
6.0
2.1
8.3
1.9
1.9
BFY013
BFY012
Strains
Glucose (20g/l)
Arabinose (20 g/m)
No Sugar

Fig. 16B

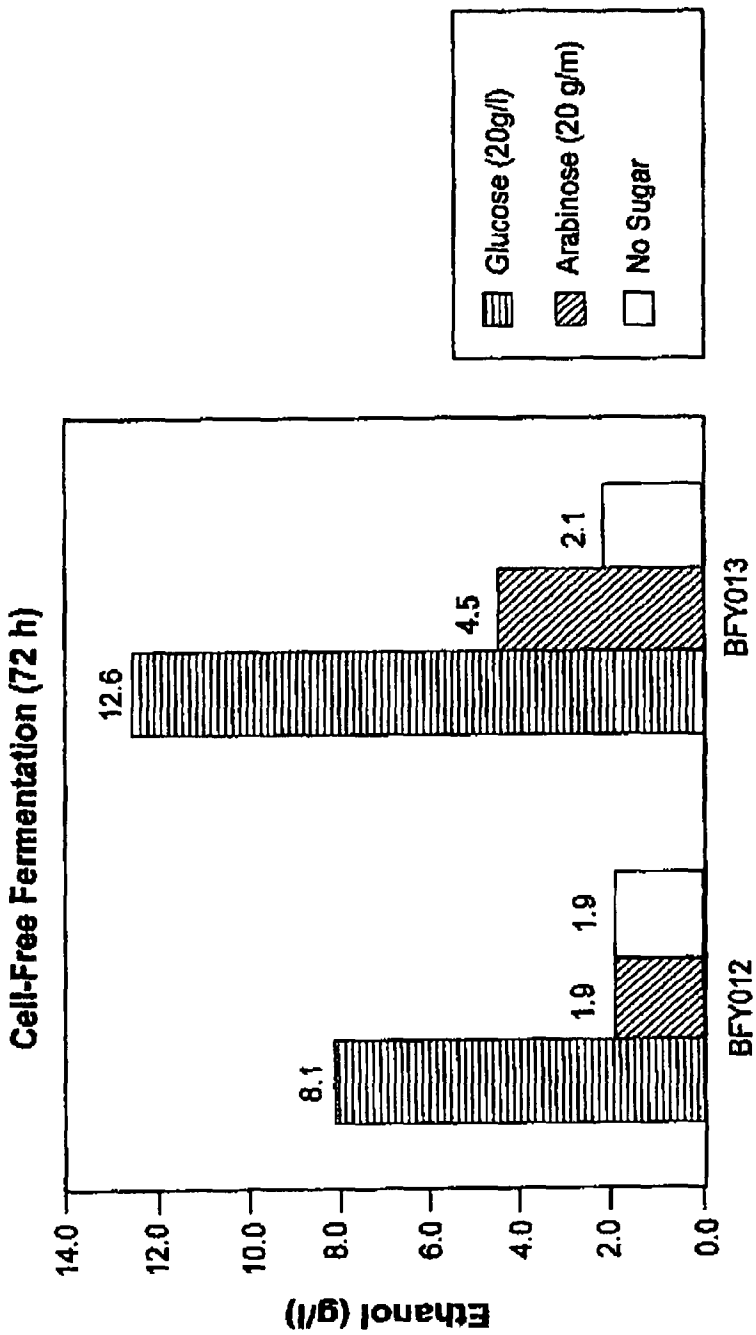
Cell-Free Fermentation (72 h)
Ethanol (g/l)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
12.6
4.5
2.1
8.1
1.9
1.9
BFY013
BFY012
Strains
Glucose (20g/l)
Arabinose (20 g/m)
No Sugar

Fig. 16C

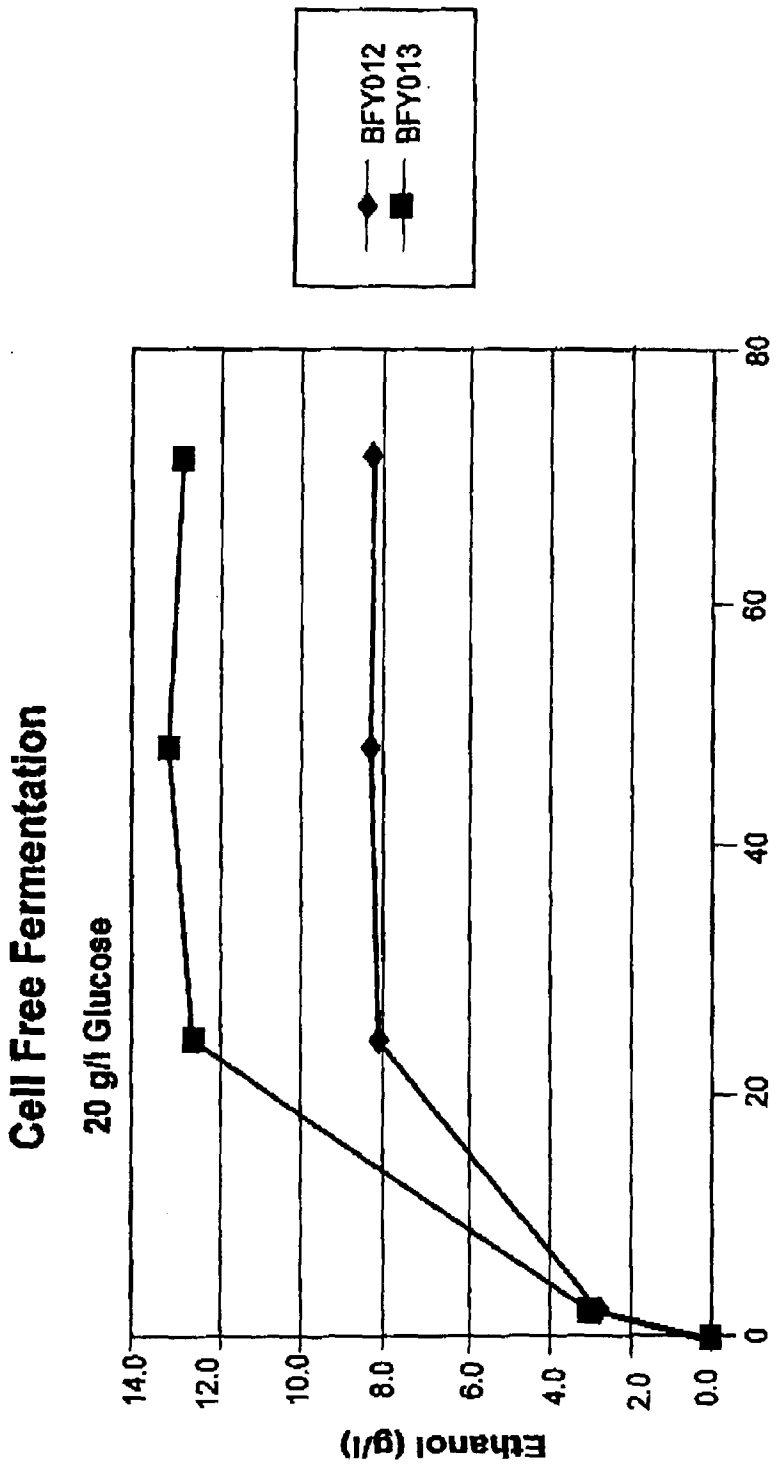
BFY012
BFY013
Cell Free Fermentation
20 g/l Glucose
Ethanol (g/l)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
20
40
60
80
Time (h)

Fig. 17A

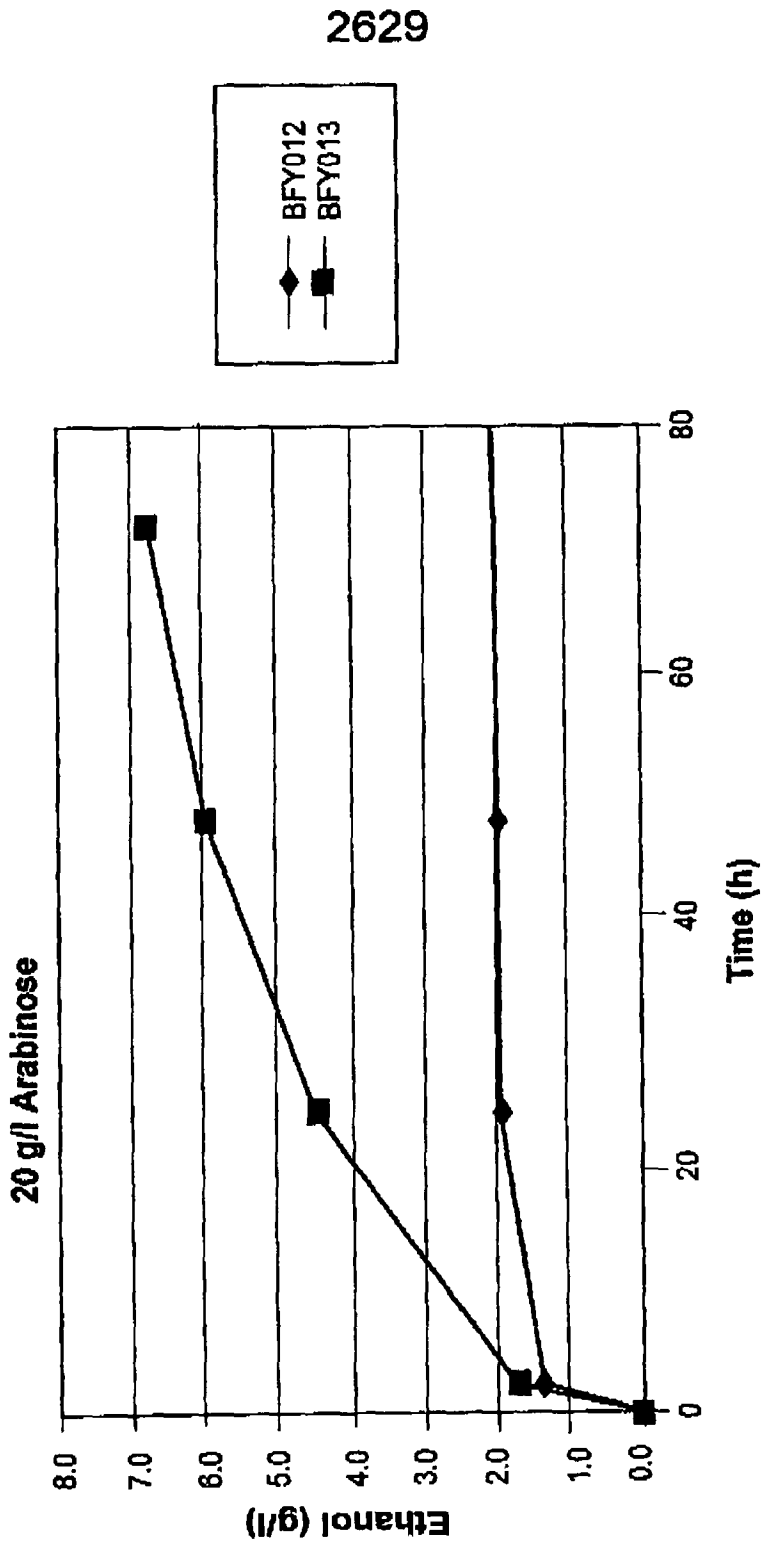
2629
BFY012
BFY013
20 g/l Arabinose
Ethanol (g/l)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
20
40
60
80
Time (h)

Fig. 17B

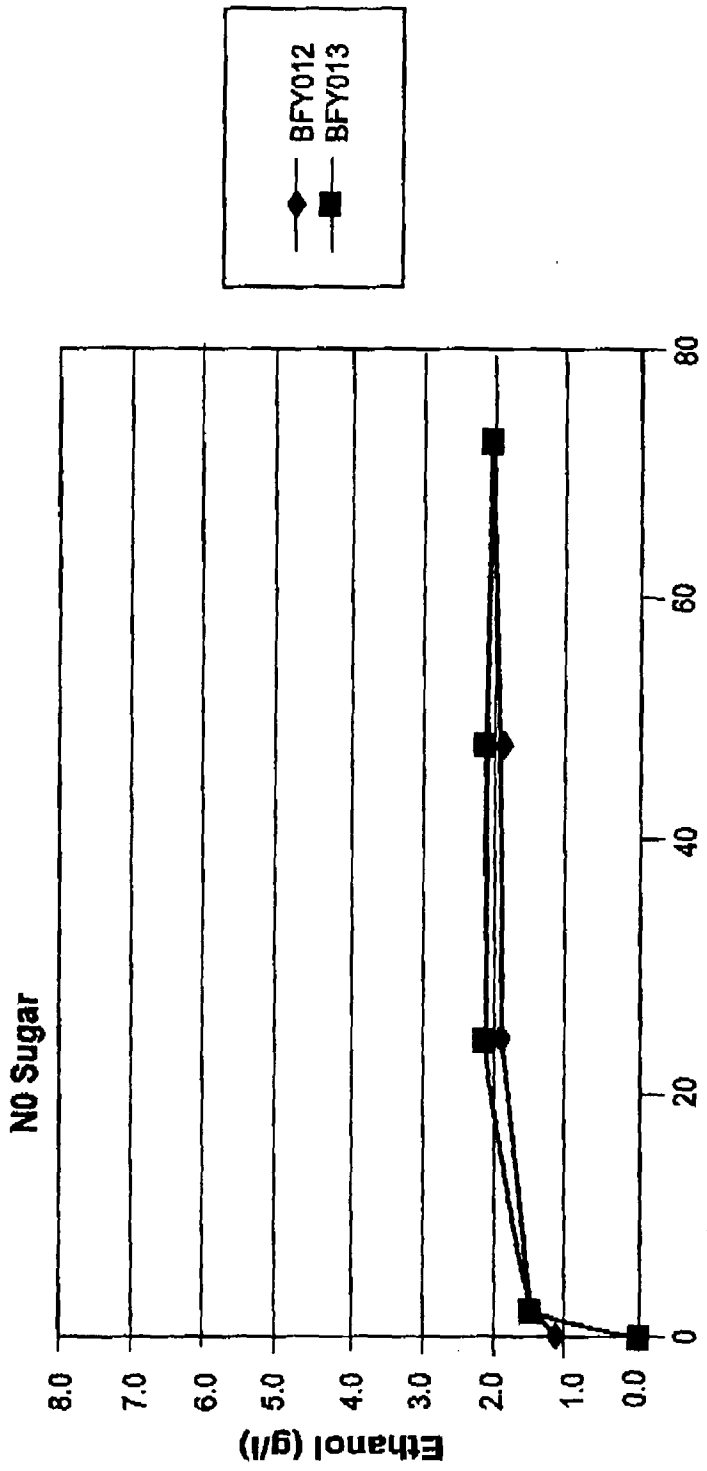
BFY012
BFY013
N0 Sugar
Ethanol (g/l)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
20
40
60
80
Time (h)

Fig. 17C

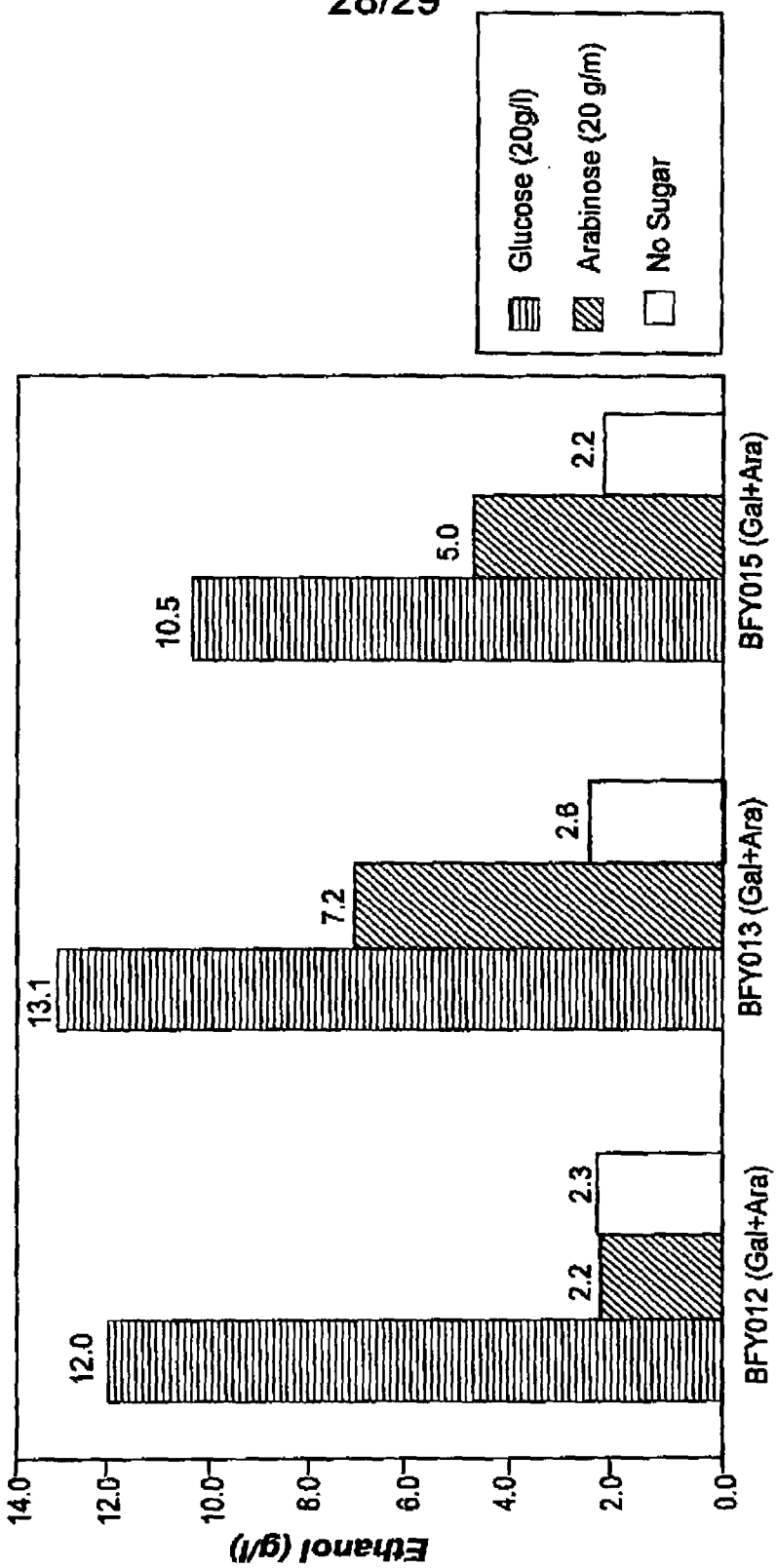
Glucose (20g/l)
Arabinose (20 g/m)
No Sugar
Ethanol (g/l)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
12.0
2.2
2.3
13.1
7.2
2.8
10.5
5.0
2.2
BFY012 (Gal+Ara)
BFY013 (Gal+Ara)
BFY015 (Gal+Ara)
STRAIN

Fig. 18

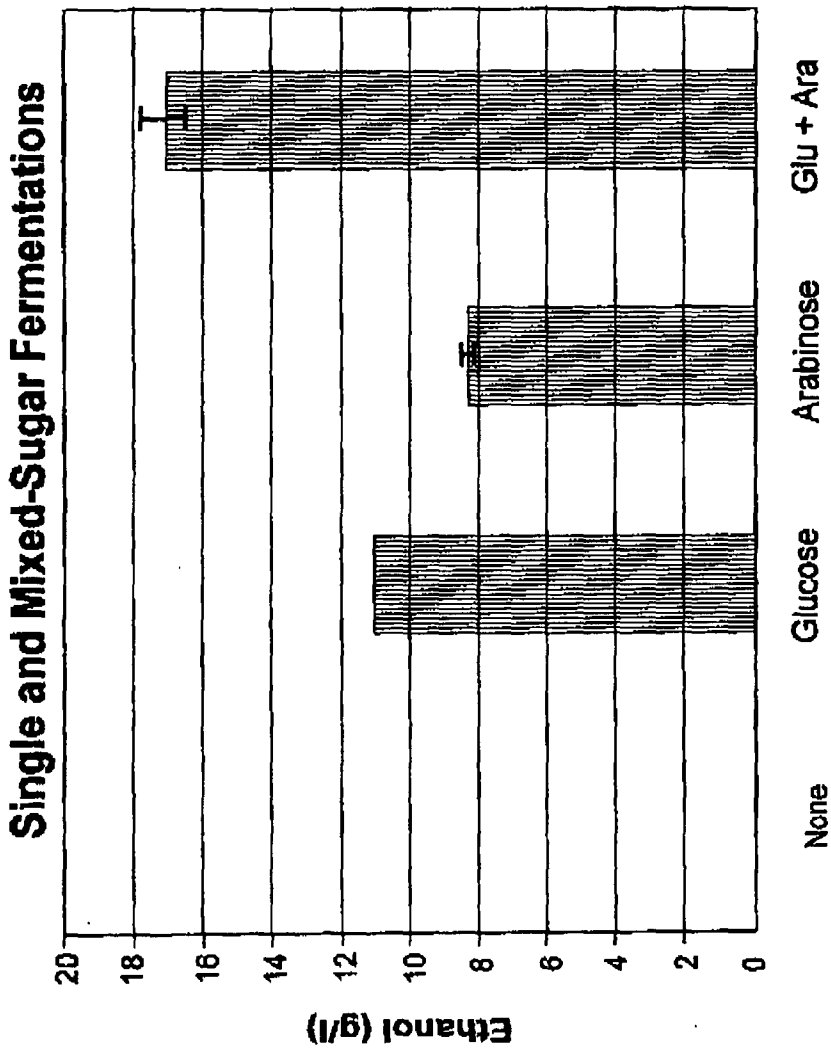
Single and Mixed-Sugar Fermentations
Ethanol (g/l)
0
2
4
6
8
10
12
14
16
18
20
None
Glucose
Arabinose
Glu + Ara
Sugar Fermented

Fig. 19