



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/54, 15/55, C12P 7/10, C12N 9/12, 9/42, 1/20, 15/01, C12P 7/06	A1	(11) International Publication Number: WO 98/45451 (43) International Publication Date: 15 October 1998 (15.10.98)
(21) International Application Number: PCT/US98/06331 (22) International Filing Date: 31 March 1998 (31.03.98) (30) Priority Data: 08/834,901 7 April 1997 (07.04.97) US (71) Applicant: UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INC. [US/US]; 223 Grinter Hall, Gainesville, FL 32611 (US). (72) Inventors: INGRAM, Lonnie, O.; 3132 N.W. 57th Terrace, Gainesville, FL 32606 (US). XIAOKUANG, Lai; 329 University Village No. 1, Gainesville, FL 32603 (US). MONIRUZZAMAN, Mohammed; 3500 Windmeadows Boulevard No. 28, Gainesville, FL 32607 (US). YORK, Sean, W.; 2231 N.W. 1st Avenue, Gainesville, FL 32603 (US). (74) Agents: ELMORE, Carolyn, S. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02173 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: RECOMBINANT MICROORGANISMS CAPABLE OF FERMENTING CELLOBIOSE		
(57) Abstract This invention relates to a recombinant microorganism which expresses pyruvate decarboxylase, alcohol dehydrogenase, Klebsiella phospho- β -glucosidase and Klebsiella (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II, wherein said phospho- β -glucosidase and said (phosphoenolpyruvate-dependent phosphotransferase) cellobiose-utilizing Enzyme II are heterologous to said microorganism and wherein said microorganism is capable of utilizing both hemicellulose and cellulose hydrolysates, including cellobiose, in the production of ethanol.		

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RECOMBINANT MICROORGANISMS CAPABLE OF FERMENTING CELLOBIOSE

BACKGROUND OF THE INVENTION

Most fuel ethanol is currently produced from hexose sugars in corn starch or cane syrup utilizing *S. cerevisiae* or *Z. mobilis*. However, such sugars are a relatively expensive source of biomass sugars and have competing value as foods. Alternatively, a major and cheap, renewable source of biomass is present in waste paper and yard trash from landfills, in the form of lignocellulose. Lignocellulose is primarily a mixture of cellulose, hemicellulose, and lignin. Cellulose is a homopolymer of glucose, while hemicellulose is a more complex heteropolymer comprised not only of xylose, which is its primary constituent, but also of significant amounts of arabinose, mannose, glucose and galactose. It has been estimated that microbial conversion of the sugar residues present in this abundant source of biomass (waste paper and yard trash) could provide over ten billion gallons of ethanol.

Recombinant microorganisms are known which can effectively ferment the mixture of sugars, formed by the hydrolysis of hemicellulose, into ethanol. See, for example, United States Patent Nos. 5,028,539 to Ingram et al., 5,000,000 to Ingram et al., 5,424,202 to Ingram et al., 5,487,989 to Fowler et al., 5,482,846 to Ingram et al., 5,554,520 to Fowler et al., 5,514,583 to Picataggio, et al., and copending applications having U.S.S.N 08/363,868 filed on December 27, 1994, U.S.S.N. 08/475,925 filed on June 7, 1995, U.S.S.N. 08/218,914 filed on March 28, 1994 the teachings of all of which are

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hereby incorporated by reference, in their entirety.
Likewise, these patents and applications describe
recombinant microorganisms that can ferment the product
of both the complete and partial hydrolysis of
5 cellulose, namely glucose and the disaccharide,
cellobiose into ethanol.

However, it would be highly advantageous to develop
a single organism which could utilize both hemicellulose
hydrolysates and cellulose hydrolysates, particularly
10 the disaccharide, cellobiose, in the process of
producing ethanol through fermentation in high yields.

SUMMARY OF THE INVENTION

The invention is based upon the discovery that the
insertion of a *Klebsiella oxytoca* *cas AB* operon into an
15 ethanologenic microorganism, such as *Escherichia coli*
K011 or *Zymomonas mobilis* CP4, provides an improved
cellobiose transport system, thereby providing a
recombinant microorganism with an improved ability to
ferment cellulosic materials to ethanol. The *K.*
20 *oxytoca cas AB* operon encodes a (phosphoenolpyruvate-
dependent phosphotransferase system) cellobiose-
utilizing Enzyme II and phospho- β -glucosidase.

Thus, in one embodiment, the invention described
herein relates to novel recombinant ethanologenic
25 microorganisms which can effectively transport
cellobiose, thereby permitting the microorganism to
utilize both hemicellulose and cellulose in the
production of ethanol. The microorganisms can be
characterized by a heterologous isolated nucleic acid
30 molecule *K. oxytoca cas AB* operon which encodes a
(phosphoenolpyruvate-dependent phosphotransferase

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system) cellobiose-utilizing Enzyme II and phospho- β -glucosidase. The cellobiose-utilizing Enzyme II is obtained from the phosphoenolpyruvate-dependent phosphotransferase system. The microorganisms are preferably organisms which are capable of fermenting xylose, glucose or both to ethanol. The invention further relates to isolated and/or recombinant nucleic acid molecules which encode the *K. oxytoca* *cas AB* operon, including homologs, active fragments or mutants thereof, the proteins encoded by these isolated and/or recombinant nucleic acid molecules, the plasmids containing the *K. oxytoca* *cas AB* operon, and the methods of using these novel recombinant organisms in the production of ethanol.

In one aspect, the present invention relates to recombinant microorganisms which express pyruvate decarboxylase (also referred to as *pdc*), alcohol dehydrogenase (also referred to as *adh*), *Klebsiella* phospho- β -glucosidase and *Klebsiella* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II. In general, the recombinant microorganisms express the above, at a sufficient functional level so as to facilitate the production of ethanol as a primary fermentation product, in high yields.

The *Klebsiella* phospho- β -glucosidase and *Klebsiella* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II, are heterologous to (i.e. foreign to) the recombinant microorganism, whereas, the pyruvate decarboxylase and alcohol dehydrogenase can be either native to or heterologous to the recombinant microorganism.

In specific embodiments, the pyruvate decarboxylase and/or alcohol dehydrogenase, of the recombinant microorganisms, are encoded by nucleic acid molecules of *Zymomonas* origin. In more specific embodiments, the
5 pyruvate decarboxylase and/or alcohol dehydrogenase, of the recombinant microorganisms, have the same or substantially the same amino acid sequence as the corresponding enzyme as it would be expressed by *Zymomonas mobilis* (hereinafter *Z. mobilis*).

10 In certain embodiments, the *Klebsiella* phospho- β -glucosidase and/or the *Klebsiella*(phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II, are encoded by a nucleic acid molecule of *Klebsiella oxytoca* origin. In other
15 embodiments, the phospho- β -glucosidase has the same or substantially the same amino acid sequence as *Klebsiella oxytoca* phospho- β -glucosidase. In further embodiments, the (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II, has the same or
20 substantially the same amino acid sequence as *Klebsiella oxytoca* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II.

A second aspect of the invention relates to a recombinant microorganism comprising heterologous
25 nucleic acid molecules encoding a *Zymomonas* pyruvate decarboxylase, a *Zymomonas* alcohol dehydrogenase, a *Klebsiella* phospho- β -glucosidase and a *Klebsiella* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II, wherein said
30 molecules are expressed at levels sufficient to convert cellobiose to ethanol. In certain embodiments, the microorganism has been further mutated, for example,

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spontaneously or from contact with a mutagen. In an additional embodiment, the mutated microorganism has been subjected to an enrichment selection, for example, in cellobiose-medium, according to methods generally
5 known in the art and described herein and in copending applicaton U.S.S.N. 08/363,868 filed Decembr 27, 1994 and Attorney Docket No. UF97-02 filed April 7, 1997 which are incorporated herein by reference.

In one preferred embodiment of this aspect of the
10 invention, the *Zymomonas* is *Zymomonas mobilis*. In another preferred embodiment, the *Klebsiella* is *Klebsiella oxytoca*.

In a specific embodiment, the recombinant microorganism comprises heterologous nucleic acid
15 molecules encoding *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase and *Klebsiella oxytoca* phospho- β -glucosidase and (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II. In further specific embodiments,
20 the heterologous nucleic acid molecules are inserted into the microorganism as a single plasmid. In particular embodiments, the heterologous nucleic acid molecules which are inserted into the microorganism as a single plasmid are under a common regulatory control
25 which can be either endogenous to or heterologous to the microorganism. In particular embodiments, the heterologous nucleic acid molecules which are inserted into the microorganism as the single plasmid are located on a plasmid in the microorganism. In an alternative
30 embodiment, the heterologous nucleic acid molecules which are inserted into the microorganism as the single

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plasmid are chromosomally integrated in the microorganism.

In yet another particular embodiment, the heterologous nucleic acid molecules encoding *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase are inserted into the recombinant microorganism in a separate plasmid from the heterologous nucleic acid molecules encoding *Klebsiella oxytoca* phospho- β -glucosidase and (phosphoenolpyruvate-dependent phospho-transferase system) cellobiose-utilizing Enzyme II. In a specific embodiment, at least one of the heterologous nucleic acid molecules inserted in the separate plasmids is under regulatory control which is endogenous to the microorganism. In another specific embodiment, at least one of the heterologous nucleic acid molecules inserted in the separate plasmids is under regulatory control which is heterologous to the microorganism. In further embodiments, at least one of the heterologous nucleic acid molecules inserted in the separate plasmids is located on a plasmid in the microorganism, and alternatively at least one of the heterologous nucleic acid molecules inserted in the separate plasmids is chromosomally integrated in the microorganism.

The availability of a single organism capable of efficiently converting glucose and xylose to ethanol, and transporting cellobiose into the organism for metabolism would allow improved co-fermentation, thereby eliminating the need for separation of solids and washing after hydrolysis, as would be necessary in procedures where separate fermentation is required. The elimination of these steps would lead to a significant reduction in cost. In addition, since both substrates

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(hemicellulose and cellulose) can be used to make ethanol, monomer sugar concentrations and cellulose concentrations can each be lower than required for separate fermentation and still achieve equivalent final
5 ethanol concentration. This also would be a significant cost savings and could allow for a shift from high solids reactors, which are very energy intensive and complex, to plugged flow reactors with pumping of solids as a slurry.

10 BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred
15 embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

20 Figure 1 is a comparison of growth (shaken flasks) in Luria broth containing cellobiose (50 g liter^{-1}) of the parental strain (K011 harboring plasmid pLOI1906) and mutant strain MM106. Cultures were incubated at 35°C in shaken flask (250 ml) containing 50 ml of Luria broth
25 with cellobiose (60 g liter^{-1}).

Figure 2A depicts *in vitro* comparisons of recombinant strains of *E. coli* K011 harboring the indicated plasmid, using p-ntirophenyl- β -D-glucoside as a model substrate. Cells were grown in Luria broth
30 containing glucose (50 g liter^{-1}).

Figure 2B depicts *in vitro* comparisons of *K. oxytoca* P2 grown in Luria broth without added sugar, Luria broth containing glucose (50 g liter⁻¹), and cellobiose (50 g liter⁻¹), using p-nitrophenyl- β -D-glucosid as a model substrate.

Figure 3A is a comparison of plasmid pLOI1906 and spontaneous deletions in plasmids pLOI1908, pLOI1909 and pLOI1910 which facilitated expression of the *cas* operon in *E. coli* KO11. Thick lines represent the vector, pUC 18 thin lines represent DNA derived from *K. oxytoca*.

Figure 3B shows an alignment of nucleotide sequences depicting the regions of plasmids pLOI1908, pLOI1909 and pLOI1910, which contain deletions of portions of vector DNA and of *K. oxytoca* DNA in comparison with the original plasmid pLOI1906.

Figure 4A is a graph depicting ethanol production from fermentation of cellobiose by recombinant strains of *E. coli* having the indicated plasmid.

Figure 4B is a graph representing cell growth from the fermentation of cellobiose by recombinant strains of *E. coli* KO11 having the indicated plasmid.

Figure 5 is a simultaneous saccharification and fermentation of mixed waste office paper (100 g liter⁻¹) by *K. oxytoca* P2 and *E. coli* KO11 harboring the indicated plasmids.

Figure 6 shows results of an *In vitro* assay to determine the expression of *K. oxytoca casAB* operon in recombinant *Z. mobilis* with pLOI1837. The activity was measured as the release of o-nitrophenol (ONP) from o-nitrophenyl-6-phosphate (ONPG-P). The result represents an average of three replica.

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Figure 7 shows results of an *In vitro* assay to determine the expression of *E. coli ptsHI* operon in recombinant *Z. mobilis* with pLOI1836. In this coupled assay, PTS enzyme II complex and phospho-cellobiase, expressed from recombinant *Z. mobilis* with pLOI1837, served as couplers. Cell extract from pLOI1837 recombinant [Zm(pLOI1837)] was mixed with that from Zm(pLOI1836). The activity was measured as the release of *p*-nitrophenol (PNP) from *p*-nitrophenyl- β -D-glucopyranoside (PNPG). The result represents an average of three replica.

Figure 8 depicts results of an *In vitro* comparison of overall activity of cellobiose hydrolysis in recombinant *E. coli* [DH5 α (pLOI1906)] and *Z. mobilis* [Zm(pLOI1832)]. The activity was measured as the release of *p*-nitrophenol (PNP) from *p*-nitrophenyl- β -D-glucopyranoside (PNPG). The result represents an average of three replica.

Figure 9 shows results of an *In vitro* comparison of overall activity of cellobiose hydrolysis in *K. oxytoca* and recombinant *E. coli*. The activity was measured as the release of *p*-nitrophenol (PNP) from *p*-nitrophenyl- β -D-glucopyranoside (PNPG). The result represents an average of three replica.

Figure 10 depicts estimation of the expression of *E. coli ptsHI* operon in *Z. mobilis* using *E. coli* (DH5 α) as a standard. In this coupled assay, PTS enzyme II complex and phospho-cellobiase, expressed from recombinant *Z. mobilis* with pLOI1837, were served as couplers. The activity was measured as the release of *p*-nitrophenol (PNP) from *p*-nitrophenyl- β -D-glucopyranoside (PNPG). The dash lines represent the

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activity from Zm(pLOI1832) and corresponding amount of DH5 α cell extract. The result represents an average of three replica.

Figure 11 depicts estimation of the expression of
5 *K. oxytoca casB* operon in *Z. mobilis* using recombinant
E. coli DH5 α (pLOI1906) as a standard. The activity was
measured as the release of o-nitrophenol (ONP) from o-
nitrophenyl- β -D-galactopyranoside 6-phosphate (ONPG-P).
The result represents an average of three replica.

10 Figure 12 shows results of an *In vitro* assay to
determine the effect of expression of *ptsHI* operon on
the expression of *casAB* operon in *Z. mobilis*. The
activity was measured as the release of o-nitrophenol
(ONP) from o-nitrophenyl- β -D-galactopyranoside 6-
15 phosphate (ONPG-P). The result represents an average of
three replica.

Figure 13 shows a model for cellobiose metabolism
in certain bacteria.

DETAILED DESCRIPTION OF THE INVENTION

20 *Escherichia coli* B has been previously engineered
for ethanol production from soluble sugars by, for
example, the chromosomal integration of *Zymomonas*
mobilis genes encoding pyruvate decarboxylase (also
referred to as *pdc*) and alcohol dehydrogenase (also
25 referred to as *adhB*), to produce *E. coli* K011. This
particular strain, *E. coli* K011, is included in Table 1,
along with other recombinant microorganisms previously
engineered for use in the conversion of, for example,
lignocellulose to ethanol.

30 Another recombinant microorganism included in the
Table, *K. oxytoca* M5A1 P2, is of particular interest.

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As indicated in Table 1, *K. oxytoca* M5A1 P2 is a derivative of *K. oxytoca* in which the *Zymomonas mobilis* *pdc* and *adhB* genes have been chromosomally integrated. It has been found that *K. oxytoca* M5A1 P2 can rapidly and efficiently convert cellobiose to high levels of ethanol. Cellobiose is a disaccharide obtained upon partial hydrolysis of cellulose and acts as an inhibitor of endoglucanase and exoglucanase. It is known that hydrolysis of cellobiose to monomeric sugar by β -glucosidase often limits cellulose digestion by fungal broths, due to inhibition of β -glucosidase by cellobiose. *K. oxytoca* M5A1 P2 appears to have the capacity to actively transport and metabolize cellobiose, eliminating the need for β -glucosidase and reducing end-product inhibition of cellulases by cellobiose.

TABLE 1

Bacteria (Plasmid)	Charac- teristics	Accession No. (Deposit Date)
<i>K. oxytoca</i> M5A1 (pLOI555)	Cm ^r , pet ^b	ATCC 68564
<i>K. oxytoca</i> M5A1 S1	Cm ^r , Ipet ^a	
<i>K. oxytoca</i> M5A1 S2	Cm ^r , Ipet ^a	
<i>K. oxytoca</i> M5A1 S3	Cm ^r , Ipet ^a	
<i>K. oxytoca</i> M5A1 P1	Cm ^r , Ipet ^a	
<i>K. oxytoca</i> M5A1 P2	Cm ^r , Ipet ^a	
<i>K. oxytoca</i> M5A1 B1	Cm ^r , Ipet ^a	
<i>E. coli</i> K011	frd, Cm ^r , Ipet ^a	
<i>E. coli</i> (pLOI510)	pet ^c	ATCC 68484 (11/28/90)
<i>E. coli</i> (pLOI308-10)	pet ^c	ATCC 67983 (5/15/89)
<i>E. coli</i> C4 (pLOI292)	pet ^c	ATCC 68237 (2/23/90)
<i>E. coli</i> TC4 (pLOI308-11)	pet ^c	ATCC 68238 (2/23/90)
<i>E. coli</i> TC4 (pLOI297)	pet ^c	ATCC 68239 (2/23/90)
<i>E. coli</i> TC4 (pLOI295)	pet ^c	ATCC 68240 (2/23/90)

- a Ipet refers to the integration of *Z. mobilis* *pdc* and *adhB* genes into the chromosome.
- b pet refers to the presence of *Z. mobilis* *pdc* and *adhB* genes in plasmid pLOI555.
- c pet refers to the presence of *Z. mobilis* *pdc* and *adhB* genes in the indicated plasmid.
- Cm^r is the an *E. coli* shuttle vector carrying the *cat* gene.

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A more detailed description of these and other related recombinant organisms, as well as the techniques and materials used in their preparation can be found in, for example, United States Patent Nos. 5,028,539 to Ingram et al., 5,000,000 to Ingram et al. 5,424,202 to Ingram et al., 5,487,989 to Fowler et al., 5,482,846 to Ingram et al., 5,554,520 to Fowler et al., 5,514,583 to Picataggio, et al., copending applications having U.S.S.N 08/363,868 filed on December 27, 1994, U.S.S.N. 08/475,925 filed on June 7, 1995, U.S.S.N. 08/218,914 filed on March 28, 1994, U.S.S.N. 08/834,900 filed on April 7, 1997 and U.S.S.N. 08/833,435 filed on April 7, 1997 and standard texts such as, Ausubel et al., *Current Protocols in Molecular Biology*, Wiley-Interscience, New York (1988) (hereinafter "Ausubel et al."), Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory Press (1992) (hereinafter "Sambrook et al.") and *Bergey's Manual of Systematic Bacteriology*, William & Wilkins Co., Baltimore (1984) (hereinafter "Bergey's Manual") the teachings of all of which are hereby incorporated by reference in their entirety.

The *E. coli* K011 recombinant microorganism described above, has been shown to efficiently convert to ethanol the mixture of sugars which result from the hydrolysis of hemicellulose, while the *K. oxytoca* M5A1 P2 has been shown to rapidly and efficiently convert cellulose and cellobiose to high levels of ethanol.

The invention is based upon the discovery that the insertion of the *Klebsiella oxytoca* *cas AB* operon into an ethanologenic microorganism, such as *Escherichia coli* K011 or *Zymomonas mobilis* CP4, provides an improved

cellobiose transport system, thereby providing a recombinant microorganism with an improved ability to ferment cellulosic materials to ethanol.

The *K. oxytoca* *cas* AB operon of the invention
5 encodes a (phosphoenolpyruvate-dependent
phosphotransferase system) cellobiose-utilizing Enzyme
II and phospho- β -glucosidase. Appropriate regulatory
sequences can be operably linked to the coding sequence
and include, for example, enhancers, promoters (native
10 or heterologous), inducers, operators, ribosomal binding
sites and transcriptional terminators. Other species of
Klebsiella suitable for isolating the nucleic acids for
use in this invention include, for example, *Klebsiella*
pneumoniae, *Klebsiella terrigena* and *Klebsiella*
15 *planticola*.

The cellobiose-utilizing Enzyme II, as described
herein, comprises an integral membrane protein which
forms the transmembrane channel and catalyzes the
phosphorylation of cellobiose, as part of the phospho-
20 transferase system (also referred to as PTS), native to
Klebsiella oxytoca. This system can also include a
protein termed Enzyme III and/or a protein termed Enzyme
I and/or a protein termed HPr (See Stryer, *Biochemistry*,
Third Edition, W.H. Freeman and Co., New York (1988)
25 p. 959-961, the teaching of which are incorporated
herein by reference). The phosphotransferase system is
a phosphoenolpyruvate-dependent system, since the
phosphoryl donor is phosphoenolpyruvate, rather than,
for example, ATP or another nucleoside triphosphate. The
30 overall effect of the PTS in *Klebsiella oxytoca* is
transport of cellobiose inside the bacterium where it is
present in the phosphorylated form. As such, nucleic

acids encoding other proteins implicated in cellobiose transport can additionally be cloned into the host cell. For example, the nucleic acid molecule can encode active Enzyme I, Enzyme III or HPr of the PTS. Genes encoding
5 PTS systems for cellobiose metabolism have been cloned from 7 different bacteria by screening libraries with methylumbelliferyl- β -D-glucopyranoside, a chromogenic analogue of cellobiose, and are described in detail, including the techniques used, in Lai, et al., *Appl.*
10 *Environ. Microbiol.* 63: 355-363 (1997), the entire teachings of which are hereby incorporated by reference in its entirety. Phospho- β -glucosidase, as described herein, is an enzyme responsible for converting phosphorylated cellobiose into the corresponding glucose
15 monomers.

Thus, the invention described herein relates to novel recombinant ethanologenic microorganisms which can effectively transport cellobiose, thereby permitting the microorganism to utilize both hemicellulose and
20 cellulose in the production of ethanol. Cellulose, as defined herein, is a homopolymer of glucose; hemicellulose is a more complex heteropolymer comprised not only of xylose, which is its primary constituent, but also of significant amounts of arabinose, mannose,
25 glucose and galactose; and cellobiose is a disaccharide obtained upon the partial hydrolysis of cellulose.

The microorganisms can be characterized by a heterologous isolated nucleic acid molecule *K. oxytoca* cas AB operon which encodes a (phosphoenolpyruvate-
30 dependent phosphotransferase system) cellobiose-utilizing Enzyme II and phospho- β -glucosidase, described in detail above. The microorganisms are preferably

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organisms which are capable of fermenting both xylose, glucose or both to ethanol.

The invention further relates to isolated and/or recombinant nucleic acid molecules which encode the *K. oxytoca cas AB* operon, described above. The isolated nucleic acid molecule can be, for example, a nucleotide sequence, such as a deoxyribonucleic (DNA) sequence or a ribonucleic acid (RNA) sequence. The isolated nucleic acid molecule can also comprise a nucleotide sequence which results from a silent mutation. Such a nucleotide sequence can result, for example, from a mutation of the native sequence in which one or more codons have been replaced with a degenerate codon, that is, a codon which encodes the same amino acid. Such mutant nucleotide sequences can be constructed using methods which are well known in the art, for example the methods discussed by Ausubel et al. and Sambrook et al..

The isolated nucleic acid molecules can also comprise a nucleotide sequence which encodes active fragments of the *K. oxytoca cas AB* operon proteins.

The isolated nucleic acid molecules also comprise a nucleotide sequence which is homologous to the nucleotide sequence which encodes the *K. oxytoca cas AB* operon. Such a nucleotide sequence exhibits more than 80% homology with the nucleotide sequence of the *K. oxytoca cas AB* operon, preferably more than about 90% homology. Particularly preferred sequences have at least about 95% homology or have substantially the same sequence. Preparation of mutant nucleotide sequences can be accomplished by methods known in the art as described in Old, et al., *Principles of Gene Manipulation*, Fourth Edition, Blackwell Scientific

Publications (1989), in Sambrook et al., and in Ausubel et al..

The invention also relates to the active protein(s) encoded by the nucleic acid molecules described above.

5 The proteins of the invention can also be recombinant proteins produced by heterologous expression of the nucleic acid molecules which encode the *K. oxytoca* cas AB operon protein(s) or a silent mutation thereof, as discussed above. The active proteins of the invention

10 can have an amino acid sequences which are homologous to the amino acid sequences expressed by the *K. oxytoca* cas AB operon. The term "homologous", as used herein, describes a protein having at least about 80% sequence identity or homology with the reference protein, and

15 preferably about 90% sequence homology, in an amino acid alignment. Most preferably, the protein exhibits at least about 95% homology or substantially the same sequence as the disclosed sequence. A homologous protein can also have one or more additional amino acids

20 appended at the carboxyl terminus or amino terminus, such as a fusion protein.

The homologous proteins of the invention can also be non-naturally occurring. In general, a homologous protein can be a mutant protein which has a modified

25 amino acid sequence resulting from the deletion, insertion or substitution of one or more amino acid residues in the amino acid sequence to which it is referenced, for example, in this invention expressed by the *K. oxytoca* cas AB operon. Both conservative and

30 non-conservative substitutions (including deletions and insertions) can be made in the amino acid sequence. Conservative substitutions are those in which a first

amino acid residue is substituted by a second residue having similar side chain properties. An example of such a conservative substitution is replacement of one hydrophobic residue, such as valine, with another
5 hydrophobic residue, such as leucine. A non-conservative substitution involves replacing a first residue with a second residue having different side chain properties. An example of this type of substitution is the replacement of a hydrophobic
10 residue, such as valine, with an acidic residue, such as glutamic acid.

Generally, nucleotides and, therefore, amino acids which can be mutated can be identified by aligning the sequence to be mutated with homologous sequences of
15 similar function from other organisms. It is typically desirable to retain highly conserved amino acids, particularly amino acids implicated in the binding or catalytic activities of the protein.

Such amino acid sequence variants can be prepared
20 by methods known in the art. For example, the desired variants can be synthesized *in vitro* using known methods of peptide synthesis. The amino sequence variants are preferably made by introducing appropriate nucleotide changes into a DNA molecule encoding the native protein,
25 followed by expression of the mutant enzyme in an appropriate vector. These methods include site-directed mutagenesis or random mutagenesis, for example.

In yet another embodiment, the nucleic acid molecule of the present invention can be a nucleic acid
30 molecule, such as a recombinant DNA molecule, resulting from the insertion into its chain by chemical or biological means, of one or more of the nucleotide

sequences described above. Recombinant DNA includes any DNA synthesized by procedures using restriction nucleases, nucleic acid hybridization, DNA cloning, DNA synthesis or any combination of the preceding. Methods
5 of construction can be found in Sambrook et al. and Ausubel et al., and additional methods are known to those skilled in the art.

The invention also includes a plasmid or vector comprising a recombinant DNA sequence or molecule which
10 comprises one or more of the nucleic acid molecules, e.g. nucleotide sequences, of the invention, as described above. The terms "plasmid" and "vector" are intended to encompass any replication competent plasmid or vector capable of having foreign or exogenous DNA
15 inserted into it by chemical or biological means and subsequently, when transformed into an appropriate non-human host organism, of expressing the product of the foreign or exogenous DNA insert (e.g, of expressing the *K. oxytoca* *cas AB* operon of the present invention). In
20 addition, the plasmid or vector is receptive to the insertion of a DNA molecule or fragment thereof containing the gene or genes of the present invention encoding the *K. oxytoca* *cas AB* operon as described herein. Procedures for the construction of DNA plasmid
25 vectors include those described in Sambrook et al. and Ausubel et al. and others known to those skilled in the art.

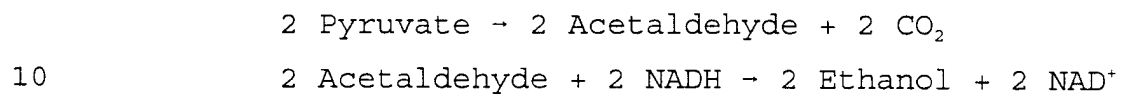
In a certain embodiment, the recombinant micro-organisms of the invention express pyruvate decarboxylase, alcohol dehydrogenase, *Klebsiella* phospho- β -glucosidase and *Klebsiella* (phosphoenolpyruvate-

30

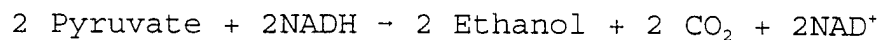
dependent phosphotransferase system) cellobiose-utilizing Enzyme II.

Alcohol dehydrogenase and pyruvate decarboxylase are enzymes required for alcoholic fermentation. The
5 net reaction for alcoholic fermentation and the intermediate reactions for the regeneration of NAD^+ , in alcoholic fermentation are as follows:

Intermediate:



Net:



Pyruvate decarboxylase is the enzyme responsible for the cleavage of pyruvate into acetaldehyde and
15 carbon dioxide, as shown in the above reaction. Alcohol dehydrogenase is the enzyme responsible for the regeneration of NAD^+ , by transferring hydrogen equivalents from NADH to acetaldehyde, thereby producing ethanol, as represented in the above reactions.

20 For purposes of this invention, the alcohol dehydrogenase activity can be provided from a gene isolated from, for example, a horse, yeast, human, insect or bacteria such as, *Zymomonas*, for example *Zymomonas mobilis*. Many alcohol dehydrogenase genes are
25 well known to those skilled in the art, as evidenced by the recitation of 252 alcohol dehydrogenase genes in the Genbank database as of March 1991 (IntelliGenetics Inc., 700 E. El Camino Drive, Mountain View, CA, 94040).
Likewise, the pyruvate decarboxylase activity can be
30 provided by a gene from *Zymomonas*, such as *Zymomonas mobilis* or by a gene which encodes the needed enzymatic

activity but which comes from corn, yeast or some other organism. At least 5 pyruvate decarboxylase genes are listed in GenBank database as of March, 1991. Therefore, one of skill in the art using standard techniques is
5 able to isolate functionally equivalent, genetically related enzymes of pyruvate decarboxylase and alcohol dehydrogenase from a variety of sources using primary information from one or more members of an enzyme family. In the case of these particular enzymes, other
10 genes can be located without sequence information, since both the pyruvate decarboxylase and alcohol dehydrogenase activity can be observed directly on aldehyde indicator plates using methods well known in the art.

15 The *Klebsiella* phospho- β -glucosidase and *Kelbsiella* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II are heterologous to (i.e. foreign to) the recombinant microorganism, whereas, the pyruvate decarboxylase and alcohol
20 dehydrogenase can be native to or heterologous to the recombinant ethanologenic microorganism. Therefore, the recombinant microorganisms can be organisms other than *Klebsiella* and are preferably organisms which are capable of fermenting both xylose, glucose or both to
25 ethanol.

For example, organisms suitable for modification in this invention include, inter alia, eukaryotic cells, such as animal cells, insect cells, fungal cells, yeasts and bacteria, particularly bacteria and yeasts.
30 Preferred host cells are bacteria and yeasts which, naturally or through mutation or recombinant engineering, encode enzymes required for the conversion

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of sugars (particularly glucose and /or xylose) to ethanol. As such, the host cells can be gram-negative or gram-positive bacteria or yeasts. Recombinant bacteria which have been engineered to convert both
5 glucose and xylose to ethanol are particularly preferred. For example, *E. coli* and other enteric bacteria of the genera *Erwinia*, like *E. chrysanthemi* are attractive because they can metabolize a variety of sugars. Other suitable hosts can be selected from the
10 broader category of gram-negative bacteria, such as species of the genus *Xanthomonas*, and from the gram-positive bacteria, such as members of the genera *Bacillus*, for example, *B. pumilus*, *B. Subtilis* and *B. coagulans*, members of the genera *Clostridium*, for
15 example, *Cl. acetobutylicum*, *Cl. aerotolerans*, *Cl. thermocellum*, *Cl. thermohydrosulfuricum* and *Cl. thermosaccharolyticum*, member of the genera *Cellulomonas* like *C. uda* and *Butyrivibrio fibrisolvens*. Acceptable yeasts, for example, are of the species of *Cryptococcus*
20 like *Cr. albidus*, *Monilia*, *Pichia stipitis*, and *Pullularia pullulans*. Another preferred microorganism is *Zymomonas mobilis*.

In specific embodiments, the pyruvate decarboxylase and/or alcohol dehydrogenase of the recombinant
25 microorganisms, are encoded by a nucleic acid molecule of *Zymomonas* origin, preferably *Z. mobilis* (See Bergey's Manual). Briefly, *Z. mobilis* is an obligatively fermentative bacterium which lacks a functional system for oxidative phosphorylation. Like the yeast
30 *Saccharomyces cerevisiae*, *Z. mobilis* produces ethanol and carbon dioxide as principal fermentation products. *Z. mobilis* has long served as an inoculum for palm wines

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and for the fermentation of Agave sap to produce pulque, an alcohol-containing Mexican beverage. The microbe also is used in the production of fuel ethanol, and reportedly is capable of ethanol production rates which are substantially higher than that of yeasts. In a further embodiment, the pyruvate decarboxylase and/or alcohol dehydrogenase expressed by the recombinant microorganisms have the same or substantially the same amino acid sequence as the corresponding enzyme as it would be expressed by *Z. mobilis*.

In certain embodiments, the *Klebsiella* phospho- β -glucosidase and/or the *Klebsiella* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II, are encoded by a nucleic acid molecule of *Klebsiella oxytoca* origin (See Bergey's Manual). In other embodiments, the phospho- β -glucosidase has the same or substantially the same amino acid sequence as *Klebsiella oxytoca* phospho- β -glucosidase. In further embodiments, the (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II, has the same or substantially the same amino acid sequence as *Klebsiella oxytoca* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II.

A second aspect of the invention relates to a recombinant microorganism comprising heterologous nucleic acid molecules encoding a *Zymomonas* pyruvate decarboxylase, a *Zymomonas* alcohol dehydrogenase, a *Klebsiella* phospho- β -glucosidase and a *Klebsiella* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II, wherein said molecules are expressed at levels sufficient to convert

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cellobiose to ethanol. In certain embodiments, the microorganism has been further mutated, for example, spontaneously or from contact with a mutagen. Suitable mutagens include radiation, e.g., ultraviolet radiation, and chemical mutagens, such as N-methyl-N'-nitroso-guanidines, hydroxylamine, ethylmethanesulfonate and nitrous acid. In an additional embodiment, the mutated microorganism has been subjected to an enrichment selection, for example, in cellobiose-medium, according to methods generally known in the art and described herein.

In one preferred embodiment of this aspect of the invention, the *Zymomonas* is *Zymomonas mobilis*. In another preferred embodiment, the *Klebsiella* is *Klebsiella oxytoca*.

In a specific embodiment, the recombinant microorganism comprises heterologous nucleic acid molecules encoding *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase and *Klebsiella oxytoca* phospho- β -glucosidase and (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II. In further specific embodiments, the heterologous nucleic acid molecules are inserted into the microorganism as the single plasmid. In particular embodiments, the heterologous nucleic acid molecules, which are inserted into the microorganism as a single plasmid, are under a common regulatory control which can be either endogenous to or heterologous to the microorganism. In particular embodiments, the heterologous nucleic acid molecules which are inserted into the microorganism as the single plasmid are located on a plasmid in the microorganism. In an alternative

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embodiment, the heterologous nucleic acid molecules, which are inserted into the microorganism as the single plasmid, are chromosomally integrated in the micro-organism as is well known in the art and described in, 5 for example, U.S. Patent No. 5,424,202 to Ingram et al., U.S. Patent No. 5,487,989 to Fowler et al. and U.S. Patent No. 5,554,520 to Fowler et al..

In yet another particular embodiment, the heterologous nucleic acid molecules encoding *Zymomonas* 10 *mobilis* pyruvate decarboxylase and alcohol dehydrogenase are inserted into the recombinant microorganism in a separate plasmid from the heterologous nucleic acid molecules encoding *Klebsiella oxytoca* phospho- β -glucosidase and (phosphoenolpyruvate-dependent phosphotransfer- 15 ase system) cellobiose-utilizing Enzyme II. In a specific embodiment, at least one of the heterologous nucleic acid molecules inserted in the separate plasmids is under regulatory control which is endogenous to the microorganism. In another specific embodiment, at least 20 one of the heterologous nucleic acid molecules inserted in the separate plasmids is under regulatory control which is heterologous to the microorganism. In further embodiments, at least one of the heterologous nucleic acid molecules inserted in the separate plasmids is 25 located on a plasmid in the microorganism, or alternatively at least one of the heterologous nucleic acid molecules inserted in the separate plasmids is chromosomally integrated in the microorganism.

In yet another aspect, the invention relates to a 30 method for making ethanol comprising the steps of contacting cellobiose with a recombinant microorganism, as described herein. In one embodiment, cellobiose can

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be contacted with a recombinant microorganism which expresses pyruvate decarboxylase, alcohol dehydrogenase, *Klebsiella* phospho- β -glucosidase and *Klebsiella* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose utilizing Enzyme II. In another embodiment, cellobiose can be contacted with a recombinant microorganism comprising heterologous nucleic acid molecules encoding a *Zymomonas* pyruvate decarboxylase, a *Zymomonas* alcohol dehydrogenase, a *Klebsiella* phospho- β -glucosidase and a *Klebsiella* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II, wherein said molecules are expressed at levels sufficient to convert cellobiose to ethanol. A detailed description of methods suitable for use in this invention can be found in, for example, United States Patent Nos. 5,028,539 to Ingram et al., 5,000,000 to Ingram et al., 5,424,202 to Ingram et al., 5,487,989 to Fowler et al., 5,482,846 to Ingram et al., 5,554,520 to Fowler et al., 5,514,583 to Picataggio, et al., and copending applications having U.S.S.N. 08/363,868 filed on December 27, 1994, U.S.S.N. 08/475,925 filed on June 7, 1995, U.S.S.N. 08/218,914 filed on March 28, 1994, U.S.S.N. 08/833,435 filed on April 7, 1997 and U.S.S.N. 08/834,900, filed on April 7, 1997, the teachings of all of which are hereby incorporated by reference, in their entirety.

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EXPERIMENTAL METHODS

BACTERIAL STRAINS AND MEDIA

E. coli KO11 (Ohta, K. et al., *Appl. Environ. Microbiol.* 57:893-900 (1991)) and *K. oxytoca* P2 (Wood, B.E. and L.O. Ingram, *Appl. Environ. Microbiol.* 58:2103-2110 (1992)) were used in all fermentation studies. These strains are derivatives of *E. coli* B and *K. oxytoca* M5A1, respectively, and contain the *Z. mobilis* genes for ethanol production (*pdc*, *adhB*) and chloramphenicol acyl transferase (*cat*). Stock cultures of KO11 and P2 were maintained on modified Luria agar (Atlas, R.M. and L.C. Parks (ed.) Handbook of Microbiological Media, CRC Press, Inc., Boca Raton, FL (1993)) containing NaCl (5 g liter⁻¹), Yeast Extract (5 g liter⁻¹), Tryptone (10 g liter⁻¹), glucose (20 g liter⁻¹), agar (15 g liter⁻¹), and chloramphenicol (0.6 g liter⁻¹). Strains KO11 and P2 are prototrophic and recombination proficient. In liquid cultures and fermentation experiments, chloramphenicol was added at a final concentration of 40 mg liter⁻¹.

Three plasmids containing genes encoding (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II (also referred to as PTS cellobiose uptake) and phospho- β -glucosidase (also referred to as cellobiose cleavage) were used in this study (Katzen, R. and D.E. Fowler, *Appl. Biochem. Biotechnol.* 45/46:697-707 (1994)): pLOI1903 containing genes from *Bacillus subtilis*, LOI1905 containing genes from *Bacteroides fibrisolvens*, and pLOI1906 containing the *K. oxytoca* *cas* AB operon. A series of mutant plasmids derived from pLOI1906 were developed and

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analyzed in this study; pLOI1908, pLOI1909, and pLOI1910.

Utilization of cellobiose (20 g liter⁻¹) was screened using MacConkey agar, M9 minimal agar, modified Luria agar containing 4-methylumbelliferyl-glucoside (20 mg liter⁻¹) and in Luria broth (Atlas, R.M. and L.C. Parks (ed.) Handbook of Microbiological Media, CRC Press, Inc., Boca Raton, FL (1993)) containing cellobiose (60 g liter⁻¹). Ampicillin (50 mg liter⁻¹) was added for plasmid selection.

10 In vitro ASSAY OF PTS ACTIVITY

The combined activity of the *casAB* phosphotransferase system and phospho-cleavage enzyme were determined using p-nitrophenyl-(pNPG) as a substrate essentially as described previously (Lai, X. et al., *Appl. Environ. Microbiol.* 63:355-363 (1997)). Overnight cultures (15 h) were harvested by centrifugation (5,000 X g, 5 minute, 4°C), washed twice, and resuspended in 50 mM NaH₂PO₄ buffer (pH 7.2) to a density of approximately 50 OD₅₅₀ ml⁻¹. Cells were disrupted by two passages through a French pressure cell at 20,000 lb in⁻². Lysates were assayed at 37°C in 50 mM NaH₂PO₄ buffer (pH 7.2) containing 5 mM MgCl₂, 2 mM pNPG and 2 mM phosphoenolpyruvate. Reactions were terminated by adding an equal volume of 1 M Na₂CO₃. After centrifugation (5,000 X g, 5 minute) to remove cell debris, p-nitrophenol was measured at 410 nm. Protein was estimated using the Bradford Reagent (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as a standard. Activities are expressed as μmoles milligram⁻¹ cell protein per minute.

GENETIC METHODS AND DNA SEQUENCING

E. coli DH5 α was used as the host for plasmid maintenance. Standard methods were employed for isolation, construction, transformation, and analysis of plasmids (Sambrook et al.) DNA was sequenced by the dideoxynucleotide chain termination method using fluorescent M13 primers (forward, 5'-CACGACGTTGTAAAACGA-3' SEQ ID NO: 1; reverse, 5'-CGATAACAATTTTCACACAGG-3' SEQ ID NO: 2) purchased from LI-COR (Lincoln, NE). A forward custom primer spanning CasA amino acid residues Phe56-Ser50 (AAAGAAGAACAGCGCATCGC SEQ ID NO: 3) was used to sequence and confirm the 5' junction between *K. oxytoca* and pUC18 by using dNTP and fluorescent-labeled ATP (LI-COR). A reverse custom primer spanning CasB amino acid residues Asn312-Leu318 (AACAAAAAAGCGCGCGGCAA SEQ ID NO: 4) was used to sequence the 3' end of *casB* and downstream region. Extension reactions were performed as previously described (Lai et al. (1997)) using a Perkin Elmer GeneAmp PCR System 9600 (Norwalk, CT) and a SequiTherm Long-Read Cycle Sequencing Kit-LC (Epicentre Technologies, Madison, WI). Products were separated and read with a LI-COR DNA Sequencer Model 4000L. Sequences were analyzed using the Wisconsin Genetics Computer Group software package (Devereux, J. et al., *Nucleic Acids Res.* 12:387-395 (1984)).

BATCH FERMENTATIONS OF CELLOBIOSE

Fermentations (350 ml volume) and analyses were carried out in modified Luria broth essentially as described previously (Beall, D.S. et al., *Biotechnol. Bioeng.* 38:296-303 (1991)) except using cellobiose (90 g liter⁻¹) as the fermentable sugar. KOH was automatically

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added to prevent broth pH from falling below pH 6.0 during fermentation (35°C, 100 rpm). Increases in pH were not controlled. Ethanol was monitored by gas chromatography. Cell mass was measured as optical
5 density at 550 nm. Using Embden-Meyerhof glycolysis and the *Z. mobilis* ethanol pathway, the maximum theoretical yield is 4 moles each of ethanol and CO₂ per mole cellobiose (0.538 g ethanol and 0.462 g CO₂ per g cellobiose).

10 SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF MIXED WASTE OFFICE PAPER

Mixed waste office paper (100 g liter⁻¹) was fermented to ethanol essentially as described previously (Brooks et al., *Biotechnology Progress* 11:619-625
15 (1995))) in 800 ml agitated (60 rpm) vessels (35°C, pH 4.9-5.2). Spezyme CP cellulase was added at a 1:100 dilution and provided approximately 1000 filter paper units liter⁻¹ (10 FPU gram⁻¹ cellulosic substrate). Fermentations were sampled at 24 hour intervals. No pH
20 control was required after the first 24 hours.

BACTERIAL STRAINS, PLASMIDS, AND GROWTH CONDITIONS

The other bacterial strains and plasmids used in this study are listed in Table 4. *Z. mobilis* strain CP4 was grown in TRG medium at 30°C. Strains of *Escherichia*
25 *coli* were grown in LB medium at 37°C (Sambrook). *Klebsiella oxytoca* P2 was grown at 30°C in LB medium containing 5% sugars. To select recombinant *Z. mobilis* and *E. coli*, 120 or 40 µg/ml chloramphenicol, respectively, were added to the media after
30 sterilization. To test the *in vivo* activity of

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cellobiose hydrolysis from recombinant *Z. mobilis*,
cellobiose analog, 4-methylumbelliferyl- β -D-
glucopyranoside (MUG) (10 μ g/ml) (Wood, T., *Methods of*
Enzymology 160:87-112 (1988)) was added to solid TRG
5 medium (15 g liter⁻¹).

MOLECULAR BIOLOGICAL TECHNIQUES

Standard procedures were used for the construction,
isolation, analysis, and transformation of recombinant
plasmids (Sambrook). PCR was performed with a Perkin-
10 Elmer Gene Amp PCR System 9600 (Perkin Elmer, Norwalk,
CT). Conjugation of plasmids from *E. coli* to *Z. mobilis*
has been described previously (Arfman, *J. Bacteriol.*
174: 7370-7378 (1992)).

In vitro ASSAY FOR THE ACTIVITY OF CELLOBIOSE HYDROLYSIS

15 Overnight cultures (approximately 20 hours) were
harvested by centrifugation (5,000 x g, 5 minutes, 4°C).
After washing twice with NaHPO₄ buffer (pH 7.2), cells
were resuspended in the same buffer and homogenized by
two passes of French pressure cell at 20,000 lb in⁻².
20 Protein concentration was determined using Bradford
Reagent (Bio-Rad Laboratories, Richmond, A) with bovine
serum albumin as a standard. The combined activity of
PTS phosphotransferase and phospho-cellobiase was
measured as described previously (Lai et al., *Appl.*
25 *Environ. Microbiol.* 63: 355-363 (1997)). The activity of
phospho-cellobiase was measured by using o-nitrophenyl
 β -D-galactopyranoside 6-phosphate (ONPG-P) as a
substrate (Fox, *PNAS, USA* 59: 988-995 (1968)). The
reaction was performed in the same buffer containing

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5 mM of MgCl_2 and 2 mM of ONPG-P. The reaction was terminated by adding equal volume of 1 M Na_2CO_3 . Activity was determined by measuring the amount of o-nitrophenol released.

5 RESULTS

ISOLATION OF CELLOBIOSE-POSITIVE MUTANTS OF KO11

Previous studies from our laboratory described the cloning of (phosphoenolpyruvate phosphotransferase) cellobiose-utilizing Enzyme II (also referred to as PTS genes for cellobiose uptake) and phospho- β -glucosidase (also referred to as genes for cellobiose cleavage) from seven microorganisms (Lai, X. et al., *Appl. Environ. Microbiol.* 63:355-363 (1997)). Although all were expressed sufficiently in DH5 α to allow cleavage of methylumbelliferyl- β -glucopyranoside (MUG), a model substrate, only three DH5 α recombinants were positive on cellobiose-MacConkey agar and grew on cellobiose-minimal medium: pLOI1903, pLOI1905, and pLOI1906. When KO11 was transformed with these three plasmids, all recombinants were positive on MUG indicator plates but none were positive on cellobiose-MacConkey agar or cellobiose-minimal medium. Plasmids were reisolated from these KO11 recombinants and transformed back into DH5 α . All were positive for cellobiose utilization in DH5 α indicating that the presence of functional *cas* genes. Restriction analysis confirmed that these plasmids appeared unaltered during passage through KO11.

Enrichment cultures of each recombinant were set up at 30°C using Luria broth containing cellobiose (10 ml broth in 1.8 x 150 mm culture tubes) to select spontaneous mutants which utilize cellobiose. These

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were diluted 100-fold every 24 hours over a three week period with continuing incubation of ancestral cultures. Several cultures with KO11 (pLOI1906) became quite dense indicating cellobiose utilization. Clones were isolated
5 from two of these by streaking on cellobiose MacConkey agar. Approximately half of the colonies from each enrichment were raised and dark red, strongly positive for cellobiose utilization. Ten positive clones (strains MM101-MM110) were selected for further testing
10 in unshaken tubes containing Luria broth containing 60 g cellobiose liter⁻¹ (Table 2). All grew to 4 times the cell density and produced 4 times as much ethanol as the unmutated KO11 (pLOI1906) or KO11(pUC18). With the mutants, broth pH declined (carbonic acid) to
15 approximately pH 5.6 while that of the parent remained nearer neutrality consistent with the absence of carbohydrate metabolism.

Repeated streaking of the mutant strains on solid medium revealed an instability in six of the ten clones
20 and these clones were discarded. The four stable clones, MM101, MM106, MM108, and MM109 grew well on M9 minimal medium containing cellobiose. Figure 1 compares the growth of the parental strain and strain MM106 on cellobiose in shaken flasks. As is characteristic for
25 ethanologenic KO11, the growth of MM106 with cellobiose was roughly linear at cell densities about 2 O.D. 550 nm.

TABLE 2. Comparison of Cellobiose-Positive Mutants of KO11 (pLOI1906)

Strain or mutant	Broth pH (24 h)	Optical Density (550 nm)	Ethanol (g liter ⁻¹)	Colony Uniformity (solid medium)
MM101	5.7	4.74	7.9	yes
MM102	5.6	4.85	7.7	no
MM103	5.7	5.00	8.0	no
MM104	5.6	4.20	7.3	no
MM105	5.6	4.05	7.0	no
MM106	5.7	4.74	7.9	yes
MM107	5.7	4.80	7.7	no
MM108	5.6	4.42	7.4	yes
MM109	5.7	4.64	7.4	yes
MM110	5.5	4.05	6.8	no
KO11 (pUC18)	6.3	1.20	1.7	yes
KO11 pLOI1906)	6.2	1.20	1.8	yes

INITIAL CHARACTERIZATION OF CELLOBIOSE-POSITIVE MUTANTS

To investigate the mechanism leading to cellobiose utilization, plasmids were isolated from strains MM101, MM106, MM108, and MM109. After transformation into KO11 and DH5 α , all recombinants retained cellobiose utilization ability indicating that plasmid mutations rather than mutation in KO11 were responsible for the acquired phenotype. Restriction analysis revealed that all four plasmids were approximately 500 base pairs smaller than the original pLOI1906. Strains MM106 and MM108 were siblings and only MM106 was retained for

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study. The plasmids from MM101, MM106, and MM109 were designated as pLOI1908, pLOI1909, and pLOI1910, respectively.

Plasmid stocks prepared using DH5 α as the host were used to transform native KO11 for further study. The stability of these plasmids in KO11 was examined by serial transfers in the absence of antibiotic selection. After 35 generations, 96% of the colonies which grew on Luria agar with glucose were also positive for cellobiose utilization when tested on cellobiose-MacConkey agar. *In vitro* expression of the *cas* operon in KO11 harboring these plasmids was evaluated by measuring the combined PTS transport and cleavage activity using pNPG as a model substrate (Figure 2A). No activity was present in control strains, KO11(pUC18) or KO11 harboring the unmutated pLOI1906. The mutant plasmids, pLOI1908, pLOI1909, and pLOI1910 were expressed in KO11 at approximately half the level measured in *K. oxytoca* P2 grown with cellobiose as an inducer (9 nmoles min⁻¹ mg protein⁻¹). Interestingly, little activity was detected in *K. oxytoca* P2 after growth in Luria broth with glucose or in Luria broth without sugar indicating that the native *cas* operon requires cellobiose for induction (Figure 2B).

GENETIC ANALYSIS OF MUTATIONS FACILITATING *casAB* EXPRESSION

Mapping with restriction endonuclease enzymes identified a deletion in the 5'-end of the *K. oxytoca* insert of all three plasmids (loss of the *EcoRI* site in the vector) while the 3'-end appeared unaltered (Figure 3A). The lack of deletions at the 3'-end of *casB* or the

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junction between the *K. oxytoca* insert and the vector was confirmed by sequence analysis.

Two patterns of deletion were found in the 5'-end which differed by only two base pairs (Figure 3B).

5 pLOI1908 and pLOI1909 were shortened by 442 base pairs of *K. oxytoca* DNA and 37 base pairs of vector DNA in comparison to the original plasmid, pLOI1906. The deletion in pLOI1910 eliminated 441 base pairs of *K. oxytoca* DNA and 38 base pairs of vector DNA. Thus the
10 results from both recombination events were essentially identical, deleting the incomplete *casR*; putative *casAB* promoter and operator regions, and a stem-loop region described Lai et al., (1997). After deletion, the *lac* Shine-Dalgarno sequence resided only a few base pairs
15 upstream from the *casA* Shine-Dalgarno region. Expression of *casAB* in pLOI1908, pLOI1909, and pLOI1910 is dependent upon the *lac* promoter (vector).

From these results, the expression of *casAB* from the native promoter (and upstream *lac* promoter) is more
20 tightly controlled in KO11, a derivative of *E. coli* B, than in DH5 α . The basis of this control may be the palindromic sequence and operator region which are presumed to require binding of an anti-terminator protein+cellobiose for expression in *K. oxytoca*. The
25 two independent deletions leading to increased expression in KO11 eliminated this regulatory region rather precisely. The resulting plasmids also retained the *lac* and *cas* Shine-Dalgarno regions in close proximity which may facilitate increased translation.
30 Recent studies have identified surprising differences in Sigma factors among K12 strains of *E. coli* (Jishage, M. et al., *J. Bacteriol.* 179: 959-963). It is possible

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that variations in Sigma factors or other regulatory proteins may be responsible for the differences in *K. oxytoca* *cas* expression between *E. coli* DH5 α and KO11 (*E. coli* B derivative).

5 FERMENTATION OF CELLOBIOSE TO ETHANOL

Ethanol production from cellobiose was examined using KO11 harboring native pLOI1906 and *in vivo* deletion mutants (pLOI1908, pLOI1909, and pLOI1910) with increased *casAB* expression as biocatalysts (Figure 4).

10 Growth and ethanol production by KO11(pLOI1906) was very poor. KO11 harboring the mutated plasmids produced up to 6-fold higher cell mass, over 20-fold higher ethanol concentrations, and consumed significant amounts of base to maintain pH 6 in comparison to KO11 harboring the
15 original pLOI1906 plasmid (Table 3). Base is typically required by KO11 during sugar fermentation to maintain pH 6 due to the production of large amounts of dissolved CO₂ (carbonic acid) and small amounts of acidic fermentation products. Fermentations with the mutants
20 rapidly reached completion and achieved approximately 1 M ethanol. Ethanol yields exceeded 90% of the maximum theoretical yield from cellobiose (0.538 g ethanol gram⁻¹ cellobiose).

It is possible to estimate a minimal *in vitro* rate
25 for cellobiose uptake and hydrolysis by KO11 derivatives based on the rate of ethanol production and an estimate of cell mass. Assuming an O.D._{550 nm} of 4.0 represents approximately 1 mg milliliter⁻¹ of cell protein, the initial rate of cellobiose metabolism is 0.03 μ moles
30 min⁻¹ milligram⁻¹ protein. This demonstrated *in vivo* activity is four times higher than the *in vitro* activity

TABLE 3. Fermentation of cellobiose and mixed waste office paper to ethanol.

Bio-catalyst	Substrate (g liter ⁻¹) ^a	Cell Mass ^b (g liter ⁻¹)	Base Consumed (mmoles liter ⁻¹)	Ethanol Produced ^c (g liter ⁻¹)	Yield ^d (% theoretical)
KO11 (pLOI1908)	cellobiose (90)	3.9	63	44.6	92
KO11 (pLOI1909)	cellobiose (90)	3.4	54	44.4	92
KO11 (pLOI1910)	cellobiose (90)	3.1	40	45.4	94
KO11 (pLOI1906)	cellobiose (90)	0.3	0	1.0	2
KO11 (pLOI1908)	paper (100)	nd	6	30.4	67
KO11 (pLOI1910)	paper (100)	nd	6	32.7	72
K. oxytoca P2 ^d	paper (100)	nd	6	34.5	76

^a Results represent an average of two or more fermentations.

Paper refers to mixed waste office paper.

^b Cell dry weight.

^c The theoretical yield is 0.538 g ethanol gram⁻¹ cellobiose and 0.568 g ethanol gram⁻¹ cellulose. Mixed waste office paper contains approximately 80% cellulose, with a maximum theoretical yield from cellulose of approximately 0.454 g ethanol gram⁻¹ mixed office waste paper.

^d All other biocatalysts are derivatives of *EE. coli* B.

TABLE 4. Bacterial strains and plasmids described herein

Strain/plasmid	Genetic characteristics
Strains	
<i>E. coli</i>	
DH5 α	F Δ lacZM15 <i>recA</i>
JLT2	F Δ <i>recA13 ptsI</i>
S17-1 (λ pir)	<i>thi pro hsdR recA</i> RP4-TC::Mu-Kn::Tn7
<i>K. oxytoca</i>	
P2	Prototroph
<i>Z. mobilis</i>	
CP4	Prototroph
Plasmids	
pUC18	<i>bla amp lacI'</i> Z'
pLOI193	<i>cat tet</i>
pLOI1844	<i>cat</i>
pLOI	pUC18 containing <i>B. stearothermophilus cel</i>
pLOI	pUC18 containing <i>B. stearothermophilus ptsHI</i>
pLOI1906	pUC18 containing <i>K. oxytoca casAB</i>
pDS20	pBR322 containing <i>E. coli ptsHI</i>
pLOI1812	<i>cel Bs-ptsHI</i>
pLOI1832	<i>lacZ-P pgm-P casAB pgm-T/adhB-P Ec-ptsHI</i> <i>adhB-T</i>
pLOI1836	<i>adhB-P Ec-ptsHI adhB-T</i>
pLOI1837	<i>lacZpP pgm-P casAB pgm-T</i>
pLOI1853	<i>lacZ-P pgm-P casAB/adhB-P Ec-ptsHI</i>
pLOI1872	<i>lacZ-P pgm-P casAB/Ec-ptsHI</i>
pLOI1877	<i>lacZ-P pgm-P casAB/Ec-ptsHI</i>
pLOI1882	<i>adhB-P casAB/Ec-ptsHI</i>
pLOI1885	<i>pgm-P casAB/Ec-ptsHI</i>
pLOI1888	<i>lacZ-P casAB/Ec-ptsHI</i>

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measured in *K. oxytoca* P2 (induced with cellobiose) and ten times higher than the best *E. coli* construct, KO11(pLOI1910) using pNPG as a model substrate.

SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF MIXED

5 WASTE OFFICE PAPER

Previous studies have demonstrated the effectiveness of *K. oxytoca* p2 for the conversion of cellulosic substrates into ethanol (Brooks et al., *Biotechnology Progress* 11: 619-625 (1995)). *E. coli* KO11 derivatives (pLOI1908, pLOI1910) expressing the *cas* operon from *K. oxytoca* were almost equivalent to P2 for ethanol production from mixed waste office paper (Figure 4, Table 3). Initial rates of fermentation were similar although P2 achieved a higher final ethanol

10 concentration KO11(pLOI1910), the construct with the highest functional expression of the *K. oxytoca casAB* operon, appeared superior to KO11(pLOI1909) for the conversion of mixed waste office paper to ethanol.

15

PLASMID CONSTRUCTION

20 To engineer *Z. mobilis* to use cellobiose, a series of recombinant plasmids were made. All plasmids were constructed on the base of an *E. coli-Z. mobilis* shuttle vector, pLOI193 (Conway, *Appl. Environ. Microbiol.* 53: 235-241 (1987)) and a smaller derivative of modified

25 pLOI193, pLOI1844. pLOI1844 was constructed by deleting a *Sfi* I/*Sac* I fragment from pLOI193 and inserting a *Sac* I linker at this site. After that, a *Pst* I/*Cla* I fragment was deleted and a *Bam* HI linker was inserted. The resulting pLOI1844 was deleted entire tetracycline

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resistant gene and ColEI replicon, and retained all other genes of pLOI193.

All plasmids were constructed by using *E. coli* strain DH5 α or JLT2 as hosts. The recombinants with
5 cellobiose genes were recovered by complementing DH5 α to use cellobiose, the recombinants with *ptsHI* operon were isolated by complementing JLT2 to use fructose.

pLOI1812 contained *cel* (Lai et al., *J. Bacteriol.* 175:6441-6450 (1993) and *ptsHI* (Lai, Microbiology
10 141:1443-1449, (1995)) operons from *Bacillus stearothermophilus*. It was constructed first by inserting a NotI/SacI fragment of *cel* operon from pLOI903 (Lai et al., *Appl. Environ. Microbiol.* 63: 355-363 (1997)) into Not I/Sac I sites of pLOI193, then
15 inserting a Not I fragment with *ptsHI* operon from pLOI800 into the Not I site.

pLOI1836 contained the *E. coli ptsHI* operon which is preceded by *Z. mobilis adhB* promoter and terminated by *adhB* terminator, its orientation of transcription is
20 opposite to that of a peptide on the vector, pBluescript KSII. The *E. coli ptsHI* operon in this plasmid was isolated from genomic DNA of DH5 α by using PCR with two custom primers (5'-ATGTCGACCTATAAGTTGGGGA SEQ ID NO: 5 and 5'-ATGGATCCATGAGAGCGATGAA SEQ ID NO: 6); this PCR
25 fragment included *crr* gene and downstream region which might also function as terminator. The *Z. mobilis adhB* promoter was isolated from digestion of pLOI287 (Conway, *J. Bacteriol* 169: 2591-2597 (1987)); the *adhB* terminator was isolated from pLOI287 by using PCR with M13
30 universal forward primer and a custom primer (5'-CCATC GATATCGCCAATCTCGG SEQ ID NO: 7). To isolate and orientate the *adhB* promoter opposite to the *LacZ*

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promoter, pLOI287 was digested with *HincII* and *EcoRI*, the promoter fragment was purified and inserted into *HincII/EcoRI* sites of pBluescript KSII, then an extra *BstEII/SmaI* fragment was deleted from this plasmid to form pLOI1861; the PCR fragment of *ptsHI* operon was first treated with Klenow and inserted into *HincII* site at pUC18, the *crr* gene in this plasmid was knocked off by digesting with *AccI* and Klenow and self ligation to form pLOI1874. To purify *ptsHI* operon fragment, pLOI1874 was first treated with *SacI* and Klenow, after denaturing the enzymes, the blunted DNA was digested with *HindIII*. The purified *ptsHI* operon was then inserted into pLOI1861 at *HincII/HindIII* sites to form pLOI1866. The *adhB* terminator was isolated from pLOI287 by using PCR with one custom primer (5'-CCATCGATATCGCCA ATCTCGG SEQ ID NO: 7) and M13 forward primer. The PCR fragment was first treated with Klenow, then digested with *SallI*. This digested PCR fragment was then ligated with pBluescript IIS which was digested with *EcoRV* and *SallI*, to form pLOI1838. A DNA fragment containing *adhB* promoter and *ptsHI* operon was isolated from pLOI1866 by first digestion with *ApaI* and Klenow, then *SacI*. This fragment was inserted into pLOI1838 at *SmaI/SacI* sites to form pLOI1840. pLOI1840 was then digested with *ScaI*, shuttle vector, pLOI1844, was digested with *BamHI* and treated with Klenow. The two digests were then ligated to form pLOI1836.

pLOI1837 contained the *Klebsiella oxytoca casAB* operon; *lacZ* promoter and *Z. mobilis pgm* promoter were fused with the operon in the upstream and a *pgm* terminator in the downstream region. The *casAB* operon and *pgm* promoter were first combined into pLOI1886. For

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constructing this plasmid, pLOI1906 was digested with *KpnI* then treated with Klenow, after denaturing these enzymes, it was digested with *HindIII*. The fragment with *casAB* was then purified and inserted into

5 *HincII/HindIII* sites on pLOI685 (Yomano, *J. Bacteriol.* 175: 3926-3933 (1993)) to form pLOI1886. The *pgm* terminator was isolated by using PCR with two custom primers (5'-ACGGCCGTTGGTCTACGAATTG SEQ ID NO: 8 and 5'-AAAGCTTCGGCATTTGGCTTCGT SEQ ID NO: 9) and pLOI685 as a

10 template. This PCR fragment was directly inserted into AT vector to form pLOI1839. An *EagI/HindIII* fragment with *pgm* promoter was then purified from pLOI1839 and ligated with pLOI1886 which was digested with the same pair of enzymes, to form pLOI1843. pLOI1843 was then

15 digested with *ScaI* and ligated with shuttle vector pLOI1844 which was digested with *BamHI* and treated with Klenow to form pLOI1837.

pLOI1888 contained both *casAB* operon and *ptsHI* operon. The *casAB* operon is expressed from *lacZ*

20 promoter, *ptsHI* operon is expressed from its native promoter. A plasmid containing *E. coli ptsHI* operon, pDS20 (Saffen, *J. Biol. Chem.* 262: 16241-16253 (1987)), was digested and treated with *BamHI*, Klenow, and *ClaI* in that order after cleaning up the enzymes in each

25 previous step. The DNA fragment containing *ptsHI* operon was purified and ligated with pLOI193, which had been digested and treated with *PstI*, Klenow, and *ClaI* in the order. The resulting plasmid, pLOI1898, was then digested and treated with *ClaI* and Klenow, and ligated

30 with a *BglIII/DraI* fragment containing *casAB* operon, which was purified from the digests of pLOI1906, to form pLOI1888.

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pLOI1885 contained both *casAB* operon and *ptsHI* operon. The *casAB* had a *pgm* promoter in front and *ptsHI* used its native promoter. This plasmid was constructed by fusing DNA fragments from pLOI1898 and pLOI1886.

5 pLOI1886 was digested and treated with *SacI*, Klenow, and *BglIII* in that order; the DNA fragment containing *pgm* promoter and *casAB* operon was then purified and ligated with pLOI18908, which was digested and treated with *SfiI*, Klenow, and *SacI* in that order.

10 pLOI1882 contained both *casAB* and *ptsHI* operons. The *casAB* had an *adhB* promoter in its front and *ptsHI* used its native promoter. The *casAB* operon and *adhB* promoter were fused to form pLOI1893 by inserting a *SacI/HindIII* fragment (*SacI* was blunted by Klenow) of
15 *casAB* operon from pLOI1906 into pLOI287 on *HincII/HindIII* sites. A *SacI/BglIII* fragment (*BglIII* was blunted by Klenow) with *adhB* promoter and *casAB* operon was then purified and ligated with pLOI1898 at *SacI/SfiI* sites (*SfiI* was blunted by Klenow) to form pLOI1882.

20 pLOI1877 contained both *casAB* and *ptsHI* operons. The *casAB* operon could be expressed from *lacZ* and *pgm* promoters. The *ptsHI* operon was expressed from its native promoter. This plasmid was constructed by fusing DNA fragments from pLOI1898 and pLOI1886. pLOI1898 was
25 served as a vector and contributed *ptsHI* operon. It was prepared by digesting with *SacI*, treating with Klenow, then digesting with *NotI*. The *casAB* operon with *lacZ* and *pgm* promoter in the front was purified from pLOI1886 digests with *DraI* and *EagI*. This fragment was then
30 ligated to the prepared pLOI1898. The *DraI* end was ligated with Klenow treated *SacI* site on pLOI1898, and *EagI* end was ligated with *NotI* on pLOI1898.

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pLOI1853 contained both *casAB* and *ptsHI* operons. The *casAB* operon could be expressed from both *lacZ* and *pgm* promoters. The *ptsHI* operon was expressed from *adhB* promoter. Two operons along with the promoters were
5 first fused in pLOI1879. For constructing this plasmid, pLOI1886 was served as a vector. It was prepared by digestion with *XbaI* then treated with Klenow. A fragment containing *ptsHI* operon and *adhB* promoter was purified from digestion of pLOI1866. One of the ends of
10 this fragment had a Klenow treated *ApaI* site, another end had a blunt *SacII* site. The resulting pLOI1879 was then digested with *ScaI* and ligated to the shuttle vector pLOI1844 which was pre-treated with *BamHI* and Klenow to form pLOI1853.

15 pLOI1872 contained both *casAB* and *ptsHI* operons. Both operons could be expressed from *lacZ* and *pgm* promoters. These DNA were orientated as: *lacZ*-promoter-*pgm*-promoter-*casAB* operon-*ptsHI* operon. The *ptsHI* operon was isolated from pDS20 by using PCR with two
20 custom primers (5'-ATGTCGACCTATAAGTTGGGA SEQ ID NO: 10 and 5'-ATGGATCCATGATCTTCTTCTA SEQ ID NO: 11). This PCR fragment was treated with Klenow then ligated into pUC18 at *HincII* site to form pLOI1847. A *XbaI/HindIII* fragment containing the *ptsHI* operon was then purified
25 from pLOI1847 and ligated to pLOI1886 at *XbaI/HindII* location to form pLOI1860. pLOI1860 was then digested with *ScaI* and ligated to shuttle vector pLOI1844 which was pre-treated with *BamHI* and Klenow to form pLOI1872.

pLOI1832 also contained both *casAB* and *ptsHI*
30 operons. The *casAB* was preceded by *lacZ* and *pgm* promoters in the order, and followed by *pgm* terminator. The *ptsHI* operon was expressed from *adhB* promoter and

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followed by *adhB* terminator. To construct this plasmid, all the essential DNA fragments from pLOI1843 and pLOI1840 were first combined to form pLOI1833. A *HindIII*/*SacI* DNA fragment containing *pgm* promoter, *casAB* operon, and *pgm* terminator was purified from pLOI1843 digestion. After treatment with Klenow, this fragment was ligated into pLOI1840, which was pre-treated with *ApaI* and Klenow, to form pLOI1833. pLOI1833 was then digested with *ScaI* and ligated with shuttle vector pLOI1844 which was pretreated with *BamHI* and Klenow to form pLOI1832.

EXPRESSION OF PTS AND CELLOBIASE GENES IN *Z. mobilis*

All the plasmid constructions were conjugated from *E. coli* strain S17-1 into *Z. mobilis* CP4 strain. The plasmids were then recovered from recombinant *Z. mobilis* and transformed back to *E. coli* to confirm that the plasmids were not mutated. None of the plasmids recovered from *Z. mobilis* were mutated. After transforming into *E. coli* JLT2, the recombinant strains could use cellobiose as well as those transformed by the original plasmids, and the pattern of enzyme digestion for these plasmids recovered from recombinant *E. coli* were the same as those for the original plasmids. The high stability of foreign plasmids in *Z. mobilis* may reflect its characteristics not as mutable as other common laboratory organisms such as *E. coli* and *Bacillus subtilis*.

For the recombinant *Z. mobilis* strains to metabolize cellobiose or MUG, these substrates were first be phosphorylated and transported into the cells, then cleaved. The *Z. mobilis* recombinants were tested

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for this combined activity on the indicator plates of TRG medium containing 10 mg/L MUG. The highest activity was observed from the recombinant *Z. mobilis* with pLOI1832, which showed bright fluorescent light after
5 overnight incubation on the MUG indicator plate, and the recombinant strain with pLOI1872 showed very weak activity to hydrolyze MUG.

In vitro assay confirmed that both *K. oxytoca* *casAB* and *E. coli* *ptsHI* operons could be functionally
10 expressed in *Z. mobilis*. When assayed with ONPG-P as a substrate significant activity of phospho-cellobiase (*casB* product) was observed from the recombinant *Z. mobilis* with pLOI1837(*casAB*), while the negative control, recombinant *Z. mobilis* with the vector
15 pLOI1844, had no activity (Figure 6).

The *in vitro* measurement of activity of HPr and enzyme II (*ptsHI* products) in recombinant *Z. mobilis* was conducted indirectly by a coupled assay with PNPG as a substrate. The conventional method to measure enzyme I
20 and HPr could not be used for *Z. mobilis*, since this organism has much higher pyruvate kinase activity than other organisms. The high activity of this enzyme would therefore cover the activities of enzyme I and JPr. The indirect assay uses phospho-cellobiase as a coupler.
25 The phospho-cellobiase, one of the products from pLOI1837, had weak activity on the non-phosphorylated PNPG (Figure 7). After mixing the cell extracts from pLOI1837 and pLOI1836 recombinants, the PNPG activity increased about three times (Figure 7). This result
30 indicated that the *E. coli* *ptsHI* was functionally expressed in the pLOI1836 recombinant *Z. mobilis*. These functional HPr and enzyme I, together with enzyme II

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complex (casA product) expressed in the pLOI1837 recombinant, formed the entire PTS phosphotransferase system. This system was able to phosphorylate PNPG, resulting higher activity from phospho-cellobiase.

5 When comparing the overall activities of cellobiose hydrolysis from recombinant *E. coli* with pLOI1906 and recombinant *Z. mobilis* with pLOI1832 with PNPG as assay substrate, the activity from the recombinant *E. coli* was about 15 times higher than that from the recombinant *Z.*
10 *mobilis* (Figure 8). However, this overall activity of DH5 α pLOI1906) was only less than half of that from *K. oxytoca* P2 strain (Figure 9).

To investigate the rate limiting step of cellobiose hydrolysis in the recombinant *Z. mobilis*, the relative
15 activities of enzyme I and HPr as well as phospho-cellobiase were estimated to compare those from *E. coli* DH5 α and DH5 α (pLOI1906), which could grow on cellobiose minimal medium. The results for the enzyme I and HPr estimation were shown in Figure 10. In this coupled
20 assay, recombinant *Z. mobilis* with pLOI1837 provided with enzyme II complex and phospho-cellobiase, and its amount kept to excess and constantly. The reaction was performed for 30 minutes. As the amount of DH5 α cell extract increased, the PNP released from PNPG increased.
25 When 0.23 mg cell extract of recombinant *Z. mobilis* with pLOI1832 was used to instead DH5 α , about 0.009 μ moles PNP was released, which amount corresponded to that released from 0.59 mg DH5 α cell extract. Therefore, the enzyme I and HPr expressed in the recombinant *Z. mobilis*
30 was more than one third of those in DH5 α ($0.23/0.59 = 39\%$). The estimation of activity of phospho-cellobiase is shown in Figure 11. This activity from *Z. mobilis*

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was only less than one tenth of that from DH5 α (pLOI1906), however, was comparable with that from *K. oxytoca*.

The fully functional system for cellobiose hydrolysis includes active PTS enzyme I, HPr, enzyme II complex, and phospho-cellobiase. The results from above *in vitro* assay indicates that low overall activity of cellobiose hydrolysis in recombinant *Z. mobilis* might result from low activity of the enzyme II complex (casA product), since part of this enzyme (EIIC domain) must be integrated into the membrane in order to fold properly and to function actively. Although the phospho-cellobiase activity from recombinant *E. coli* is much higher than that from *K. oxytoca* (Figure 11), the overall activity is only less than half of that from *K. oxytoca*, indicating the *K. oxytoca* enzyme II complex might not be properly folded in the *E. coli* membrane so that it might not be as active as in the native *K. oxytoca* cells. The same problem could happen in *Z. mobilis*. Another possible reason for low overall activity might be the complementation of *E. coli* enzyme I and HPr with *K. oxytoca* cellobiose specific enzyme II complex, which would result in poor phosphorylation of cellobiose and poor transport.

Expression of heterologous genes in microbial expression systems depends on components of the system. One of the most important components is the promoter. The major difference between plasmid pLOI1977 and pLOI1872 was the promoters for the *ptsHI* operon. In pLOI1877, *ptsHI* operon was expressed from its native *E. coli* promoter. In pLOI1972, *lacZ* and *pgm* promoters were responsible for the expression of *ptsHI* operon. The

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casAB operon on both plasmids were expressed from *lacZ* and *pgm* promoters. Both plasmids recovered *ptsI* function of a *ptsI* mutant *E. coli* strain, JLT2, and permitted this mutant to grow on cellobiose minimal
5 medium. However, the recombinant *Z. mobilis* with pLOI1872 had weak activity to hydrolyze MUG, while that with pLOI1877 had no activity. These results indicated that the native promoter of *E. coli ptsHI* might not function in *Z. mobilis*.

10 Terminator is another important component in the expression. Other have reported that stem-loop at the 3' end of *gap-pgk* operon of *Z. mobilis* is a transcriptional terminator both in *Z. mobilis* and *E. coli*, required to stabilize the full-length *gap-pgk*
15 message. The 3' stem-loops have also been reported as required to block degradation by abundant 3' to 5' exoribonucleases in *E. coli* and other bacteria. In this study, the presence of stem-loops helped the expression greatly. The recombinant *Z. mobilis* with pLOI1853,
20 which did not include stem-loops for the operons, did not show any activity on the indicator plates, while that with pLOI1832, which included respective stem-loops for the operons, showed high activity to hydrolyze MUG on the indicator plates. These stem-loops might
25 function as transcriptional terminators to stabilize the messages of *casAB* and *ptsHI* operons in *Z. mobilis*. But these stem-loops may not be necessary in *E. coli*, since recombinant *E. coli* strains with these two plasmids displayed the characteristics of the two operons equally
30 well. The higher rate of transcription in *E. coli* might compensate the degradation of messages by the exoribonucleases.

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The expression of *ptsHI* operon appeared to help the expression of *casAB* operon in *Z. mobilis* (Figure 12). The difference between pLOI1832 and pLOI1837 was that pLOI1832 included both expressible *casAB* and *ptsHI* operons (Figure 10 and Figure 11), while pLOI1837 contained expressible *casAB* operon only (Figure 6). Other components on these two plasmids were the same, including same vector, location on the vector, as well as promoter and terminator for the *casAB* operon.

However, the activity of phospho-cellobiase (*casB* product) from *Z. mobilis* recombinant with pLOI1832 was more than double than that in the recombinant with pLOI1837.

SUMMARY OF RESULTS

In engineered *Z. mobilis* strain CP4(pLOI1832), *casAB* and *ptsHI* are functionally expressed as demonstrated by the use of chromogenic cellobiose analogues. 4-Methyl-umbelliferyl- β -D-glucopyranoside is transported into cell, phosphorylated, and cleaved into a chromogenic (fluorescent) product (4-methylumbelliferone) and glucose-phosphate which is readily observed on indicator plates *in vitro*, p-nitrophenyl- β -D-glucopyranoside is phosphorylated and cleaved into a chromogenic product (p-nitrophenol) and glucose-phosphate. Constructs lacking either the *ptsHI* operon or *casAB* operon do not exhibit these activities, but can be mixed *in vitro* and the activity reconstituted.

However, the overall activity of the uptake process in the best recombinant *Z. mobilis*, CP4(pLOI1832) still does not metabolize cellobiose fast enough to support

growth on this substrate in the absence of another fermentable sugar.

Activities of InzI and Hpr are estimated to be approximately 1/3 of that present in *E. coli*, an organism which effectively uses these general proteins for the transport and phosphorylation of many sugars via PTS enzymes. The *casB* product, the phospho- β -glucosidase, is expressed at a level equivalent to *K. oxytoca*, an organism which is very proficient in the fermentation of cellobiose. Table 5 summarizes the *in vivo* activity of cellobiose hydrolysis of recombinant *Zymomonas mobilis*, of the invention.

TABLE 5. *In Vivo* Activity of Cellobiose Hydrolysis of Recombinant *Zymomonas mobilis*

Plasmid Number	Genes/Promoters (P)/Terminators (T)	MUG Activity in <i>Z. mobilis</i>
pLOI1844	Vector	-
pLOI1810	<i>Bs-cel</i>	-
pLOI1812	<i>Bs-cel/Bs-ptsHI</i>	-
pLOI1836	<i>Zm-adhB-P-Ec-ptsHI-ZM-adhB-T</i>	-
pLOI1894	<i>lacZ-P-Ko-casAB</i>	-
pLOI1852	<i>lacZ-P-Zm-pgm-P-Ko-casAB</i>	-
pLOI1837	<i>lacZ-P-Zm-pgm-P-Ko-casAB-Zm-pgm-T</i>	-
pLOI1888	<i>lacZ-P-Ko-casAB/Ec-ptsHI</i>	-
pLOI1885	<i>Zm-pgm-P-Ko-casAB/Ec-ptsHI</i>	-
pLOI1882	<i>Zm-adhB-P-Ko-casAB/Ec-ptsHI</i>	-
pLOI1877	<i>lacZ-P-Zm-pgm-P-Ko-casAB/Ec-ptsHI</i>	-
pLOI1853	<i>lacZ-P-Zm-pgm-P-Ko-casAB/Zm-adhB-P-Ec-ptsHI</i>	-
pLOI1872	<i>lacZ-P-Zm-pgm-P-Ko-casAB-Ec-ptsHI</i>	+
pLOI1832	<i>lacZ-P-Zm-pgm-P-Ko-casAB-Zm-pgm-T/Zm-adhB-P-Ec-ptsHI-Zm-adhB-T</i>	+++

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EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: University of Florida Research Foundation,
Incorporated

(ii) TITLE OF INVENTION: Recombinant Organisms Capable
of Fermenting Cellobiose

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Hamilton, Brook, Smith & Reynolds, P.C.

(B) STREET: Two Militia Drive

(C) CITY: Lexington

(D) STATE: MA

(E) COUNTRY: USA

(F) ZIP: 02173-4799

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: Windows 95

(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/834,901

(B) FILING DATE: 07-APR-1997

-55-

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Elmore, Carolyn S
- (B) REGISTRATION NUMBER: 37,567
- (C) REFERENCE/DOCKET NUMBER: UF97-01 PCT

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 781-861-6240
- (B) TELEFAX: 781-861-9540
- (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CACGACGTTG TAAAACGA

18

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGATAACAAT TTCACACAGG

20

(2) INFORMATION FOR SEQ ID NO:3:

-56-

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAAGAAGAAC AGCGCATCGC

20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AACAAAAAAG CGCGCGGCAA

20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGTCGACCT ATAAGTTGGG GA

22

(2) INFORMATION FOR SEQ ID NO:6:

-57-

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGGATCCAT GAGAGCGATG AA

22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCATCGATAT CGCCAATCTC GG

22

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACGGCCGTTG GTCTACGAAT TG

22

(2) INFORMATION FOR SEQ ID NO:9:

-58-

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAAGCTTCGG CATTGGCTTC GT

22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATGTCGACCT ATAAGTTGGG A

21

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATGGATCCAT GATCTTCTTC TA

22

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CLAIMS

What is claimed is:

1. A recombinant microorganism which expresses pyruvate decarboxylase, alcohol dehydrogenase,
5 Klebsiella phospho- β -glucosidase and Klebsiella (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II, wherein said phospho- β -glucosidase and said (phosphoenolpyruvate-dependent phosphotransferase
10 system) cellobiose-utilizing Enzyme II are heterologous to said microorganism.
2. The recombinant microorganism according to Claim 1 wherein said pyruvate decarboxylase and alcohol dehydrogenase are heterologous to said
15 microorganism.
3. The recombinant microorganism according to Claim 1 wherein said pyruvate decarboxylase is encoded by a nucleic acid molecule of *Zymomonas* origin.
4. The recombinant microorganism according to Claim 1
20 wherein said alcohol dehydrogenase is encoded by a nucleic acid molecule of *Zymomonas* origin.
5. The recombinant microorganism according to Claim 1 wherein said pyruvate decarboxylase has the same or substantially the same amino acid sequence as
25 *Zymomonas mobilis* pyruvate decarboxylase.

6. The recombinant microorganism according to Claim 1 wherein said alcohol dehydrogenase has the same or substantially the same amino acid sequence as *Zymomonas mobilis* alcohol dehydrogenase.
- 5 7. The recombinant microorganism according to Claim 1 wherein said phospho- β -glucosidase is encoded by a nucleic acid molecule of *Klebsiella oxytoca* origin.
8. The recombinant microorganism according to Claim 1 wherein said (phosphoenolpyruvate-dependent
10 phosphotransferase system) cellobiose-utilizing Enzyme II is encoded by a nucleic acid molecule of *Klebsiella oxytoca* origin.
9. The recombinant microorganism according to Claim 1 wherein said phospho- β -glucosidase has the same or
15 substantially the same amino acid sequence as *Klebsiella oxytoca* phospho- β -glucosidase.
10. The recombinant microorganism according to Claim 1 wherein said (phosphoenolpyruvate-dependent
phosphotransferase system) cellobiose-utilizing
20 Enzyme II has the same or substantially the same amino acid sequence as *Klebsiella oxytoca* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II.
11. A recombinant microorganism comprising heterologous
25 nucleic acid molecules encoding a *Zymomonas* pyruvate decarboxylase, a *Zymomonas* alcohol dehydrogenase, a *Klebsiella* phospho- β -glucosidase

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and a *Klebsiella* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II wherein said molecules are expressed at levels sufficient to convert cellobiose to ethanol.

- 5 12. The recombinant microorganism according to Claim 11 wherein said *Zymomonas* is *Zymomonas mobilis*.
13. The recombinant microorganism according to Claim 12 wherein said *Klebsiella* is *Klebsiella oxytoca*.
14. The recombinant microorganism according to Claim 11
10 wherein said heterologous nucleic acid molecules contain coding sequences obtained from *Zymomonas mobilis* and *Klebsiella oxytoca*.
15. The recombinant microorganism according to Claim 14
15 wherein said heterologous nucleic acid molecules are inserted into said microorganism as a single plasmid.
16. The recombinant microorganism according to Claim 15 wherein said heterologous nucleic acid molecules are under a common regulatory control.
- 20 17. The recombinant microorganism according to Claim 16 wherein said regulatory control is endogenous to the microorganism.
18. The recombinant microorganism according to Claim 16
25 wherein said regulatory control is heterologous to the microorganism.

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19. The recombinant microorganism according to Claim 16 wherein said heterologous nucleic acid molecules are located on a plasmid in the microorganism.
20. The recombinant microorganism according to Claim 15 wherein said heterologous nucleic acid molecules are chromosomally integrated in the microorganism.
21. The recombinant microorganism according to Claim 14 wherein said heterologous nucleic acid molecules obtained from *Zymomonas mobilis* are inserted into said microorganism in a separate plasmid from said heterologous nucleic acid molecules obtained from *Klebsiella oxytoca*.
22. The recombinant microorganism according to Claim 21 wherein at least one of said heterologous nucleic acid molecules is under regulatory control which is endogenous to the microorganism.
23. The recombinant microorganism according to Claim 21 wherein at least one of said heterologous nucleic acid molecules is under regulatory control which is heterologous to the microorganism.
24. The recombinant microorganism according to Claim 21 wherein at least one of said heterologous nucleic acid molecules is located on a plasmid in the microorganism.

25. The recombinant microorganism according to Claim 21 wherein at least one of said heterologous nucleic acid molecules is chromosomally integrated in the microorganism.
- 5 26. The recombinant microorganism according to Claim 11 wherein said microorganism has been further mutated.
- 10 27. The recombinant microorganism of Claim 26 wherein said microorganism has been subjected to an enrichment selection.
28. The recombinant microorganism of Claim 26 wherein said microorganism has been contacted with a mutagen.
- 15 29. A recombinant nucleic acid molecule comprising nucleic acid molecules encoding pyruvate decarboxylase, alcohol dehydrogenase, *Klebsiella* phospho- β -glucosidase and *Klebsiella* (phosphoenolpyruvate-dependent phosphotransferase system) cellobiose-utilizing Enzyme II.
- 20 30. The recombinant nucleic acid molecule according to Claim 29 wherein said pyruvate decarboxylase and alcohol dehydrogenase are of *Zymomonas* origin.
- 25 31. A method for making ethanol comprising the steps of contacting cellobiose with a recombinant microorganism according to Claim 1.

32. A method for making ethanol comprising the steps of contacting cellobiose with a recombinant microorganism according to Claim 11.

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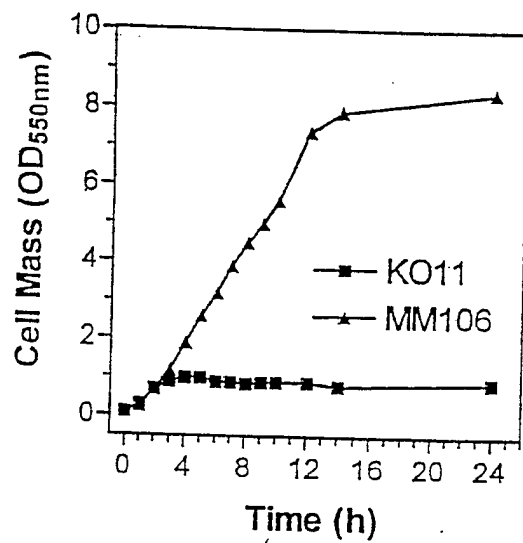


FIG. 1

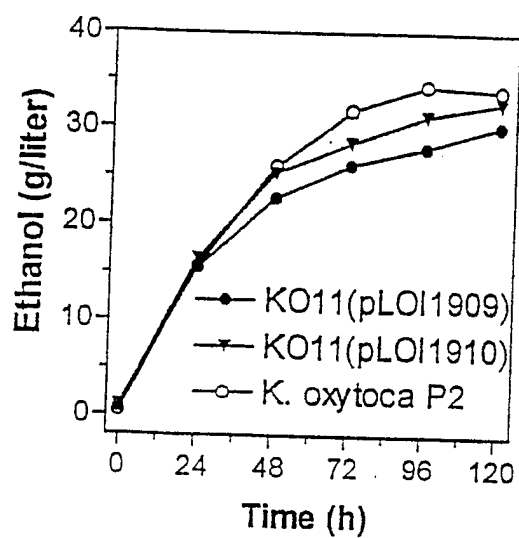


FIG. 5

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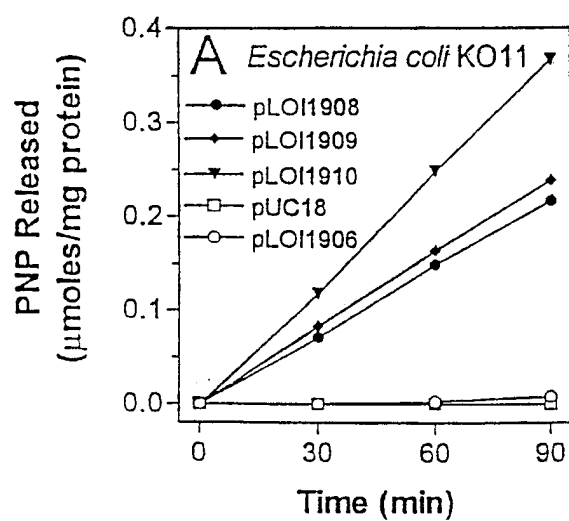


FIG. 2A

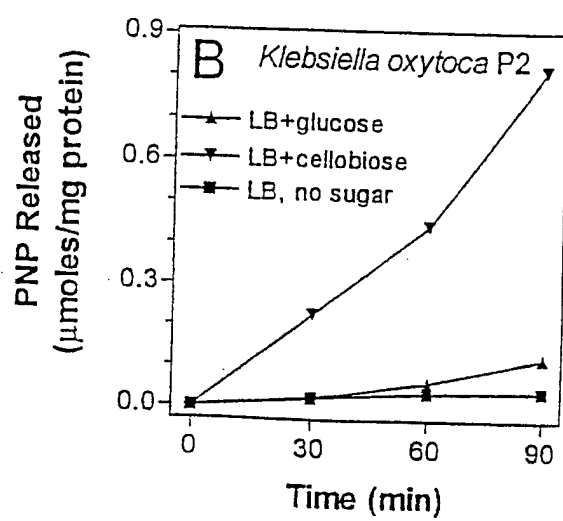


FIG. 2B

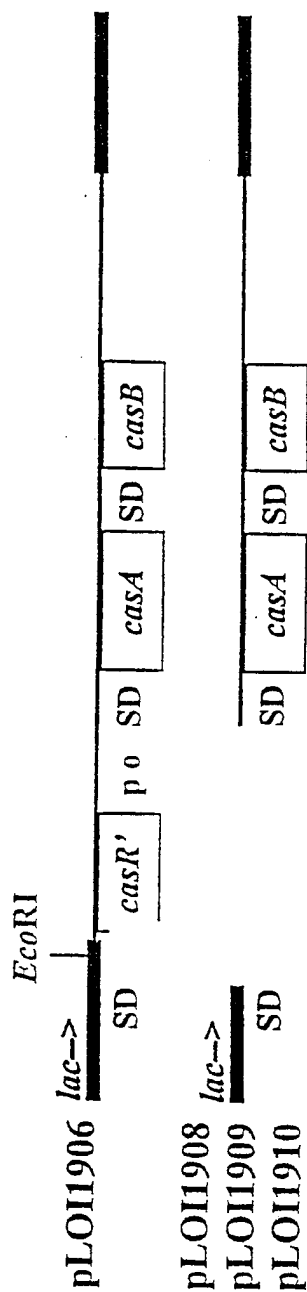


FIG. 3A

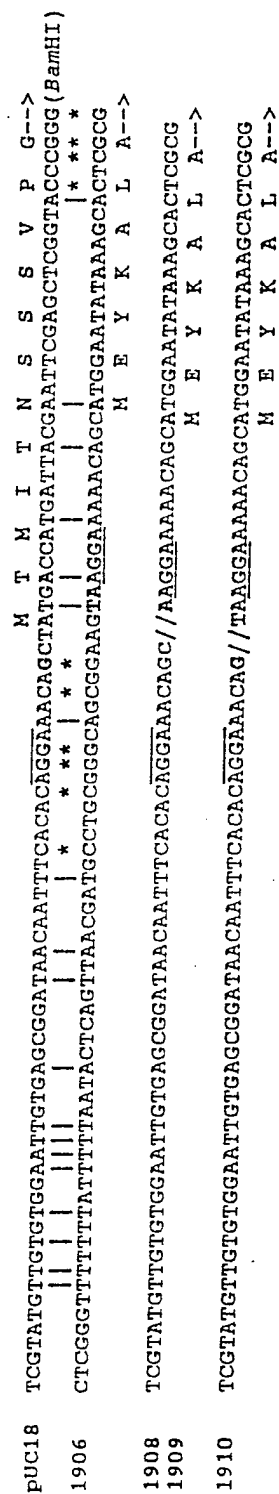


FIG. 3B

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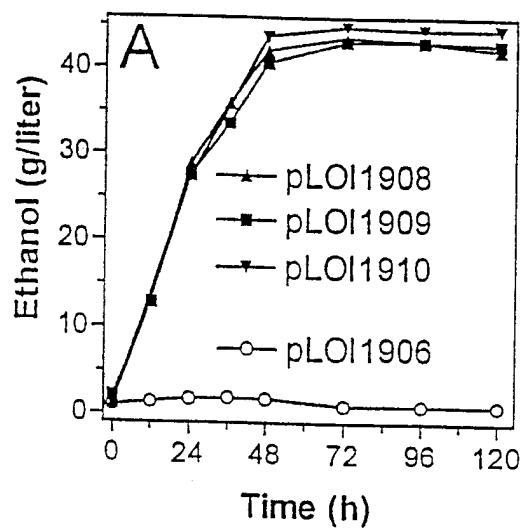


FIG. 4A

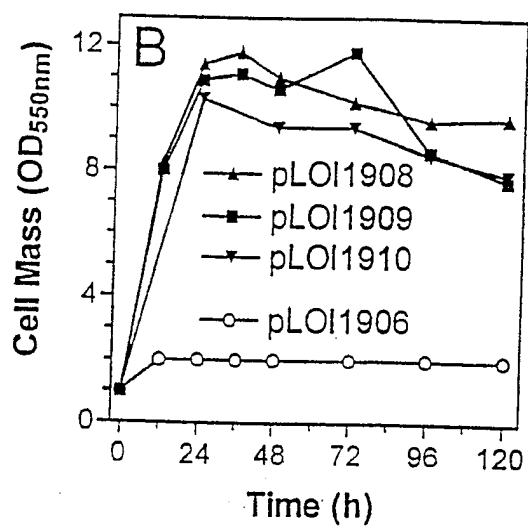


FIG. 4B

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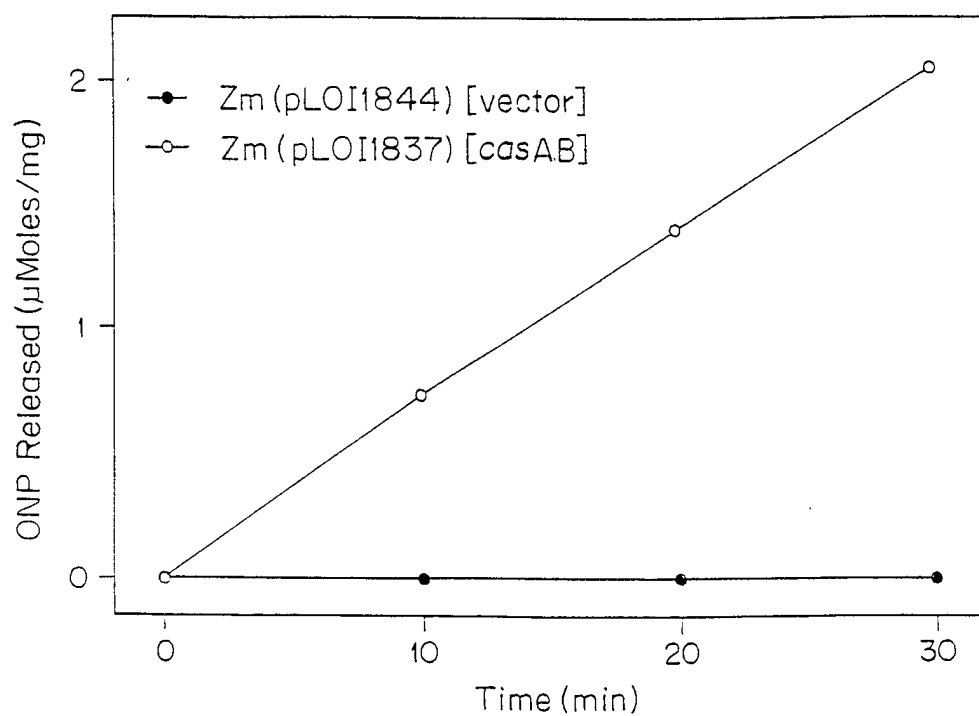


FIG. 6

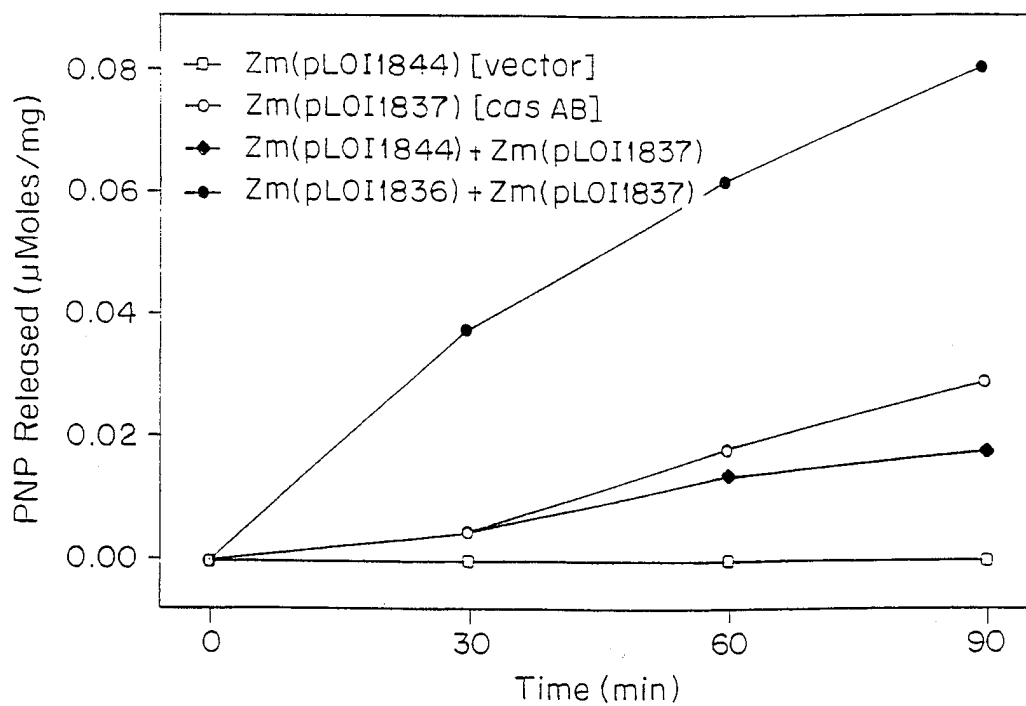


FIG. 7

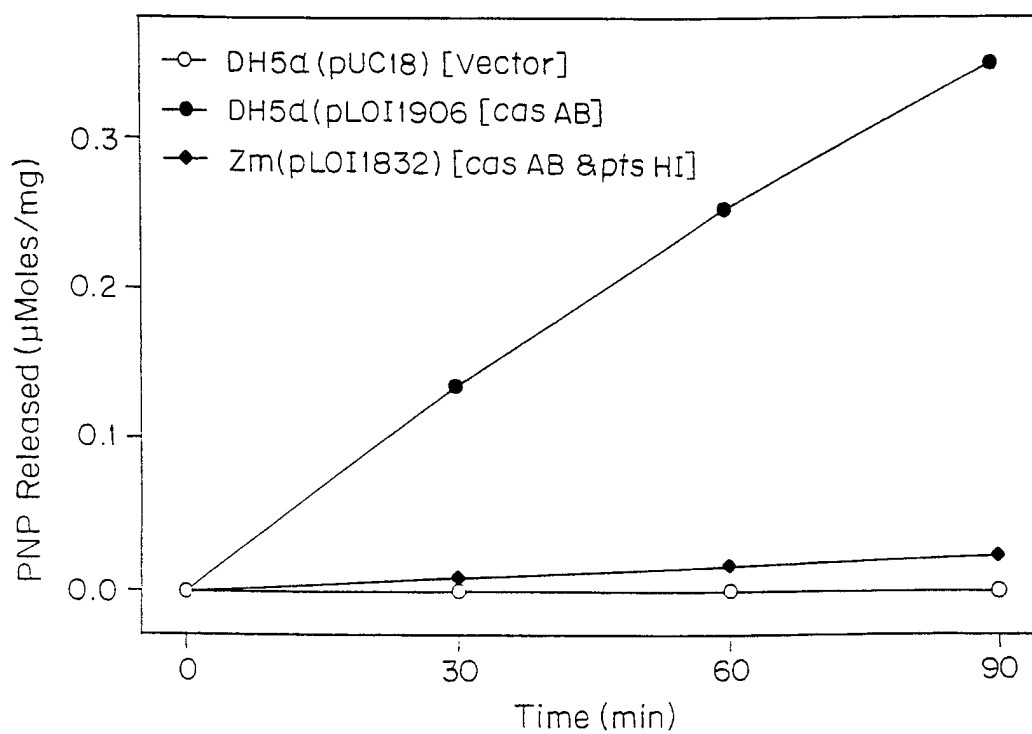


FIG. 8

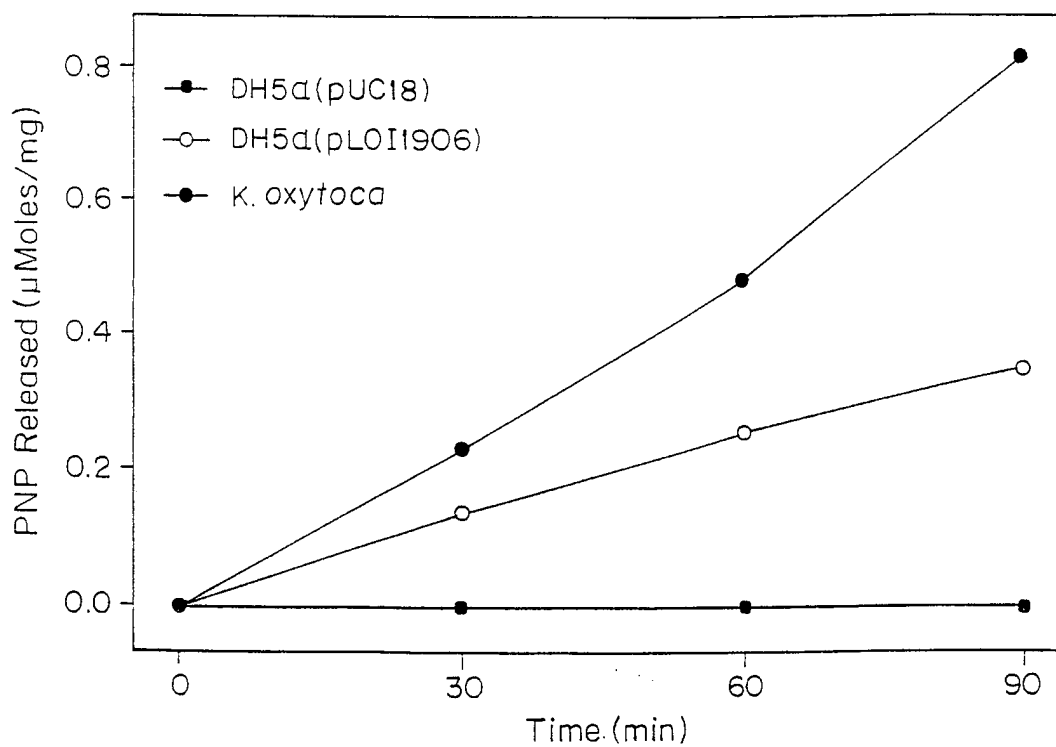


FIG. 9

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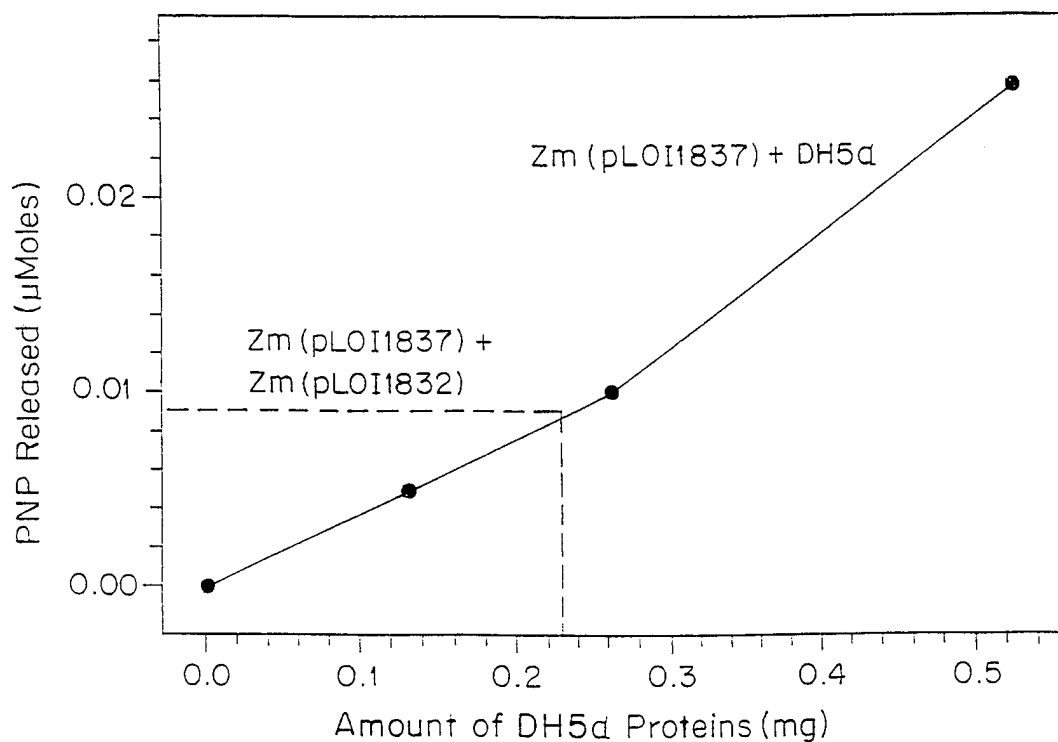


FIG. 10

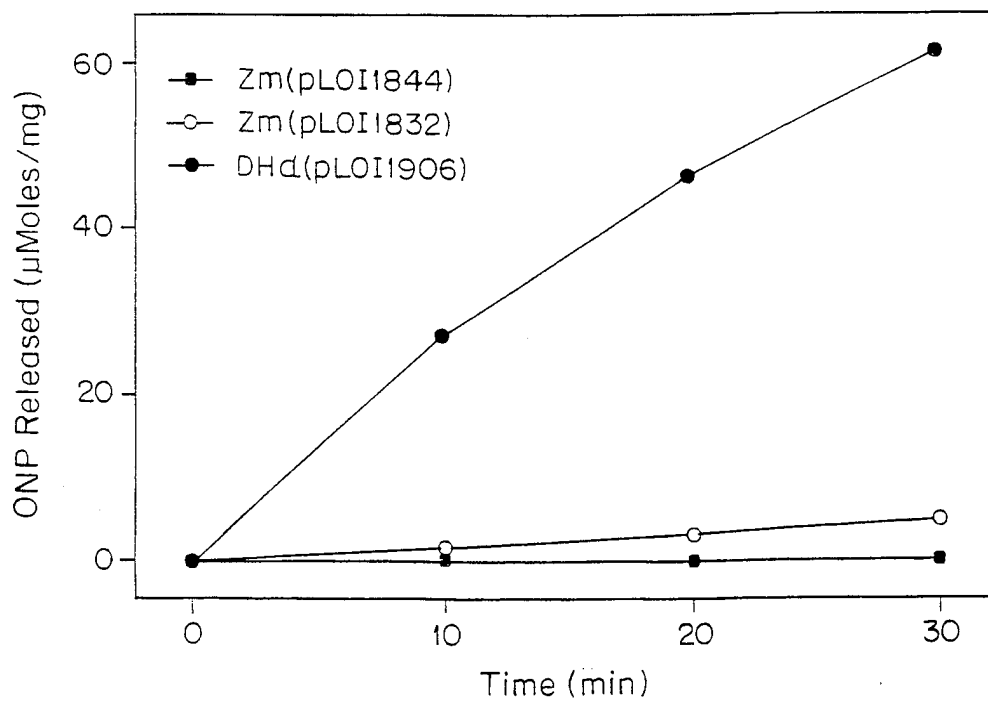


FIG. 11

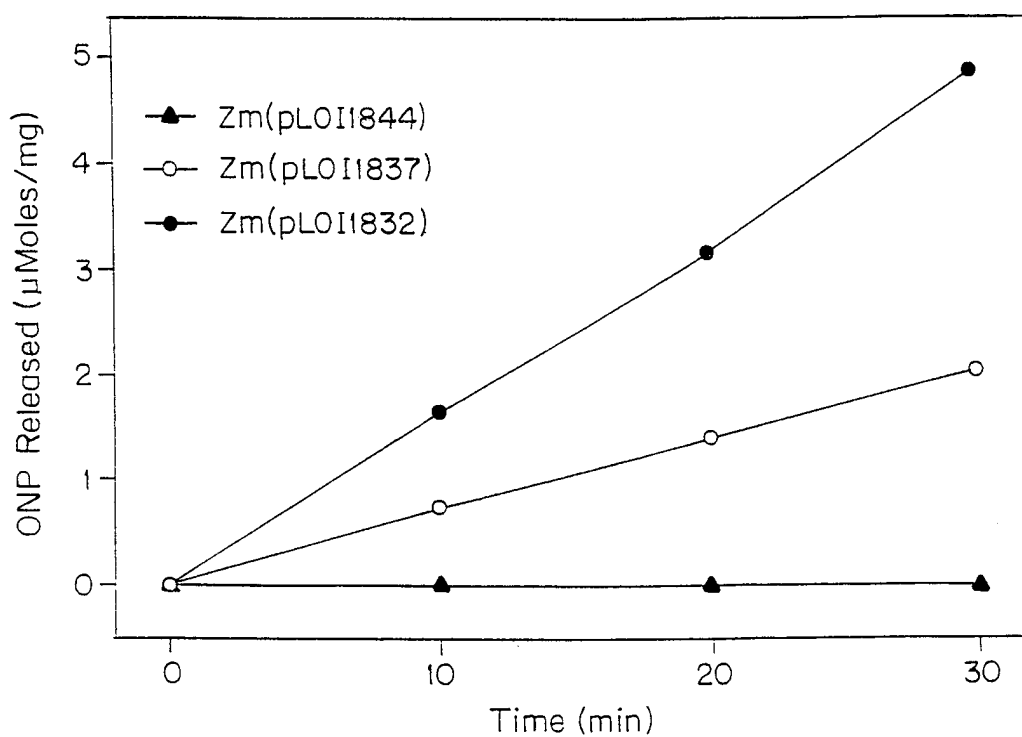


FIG. 12

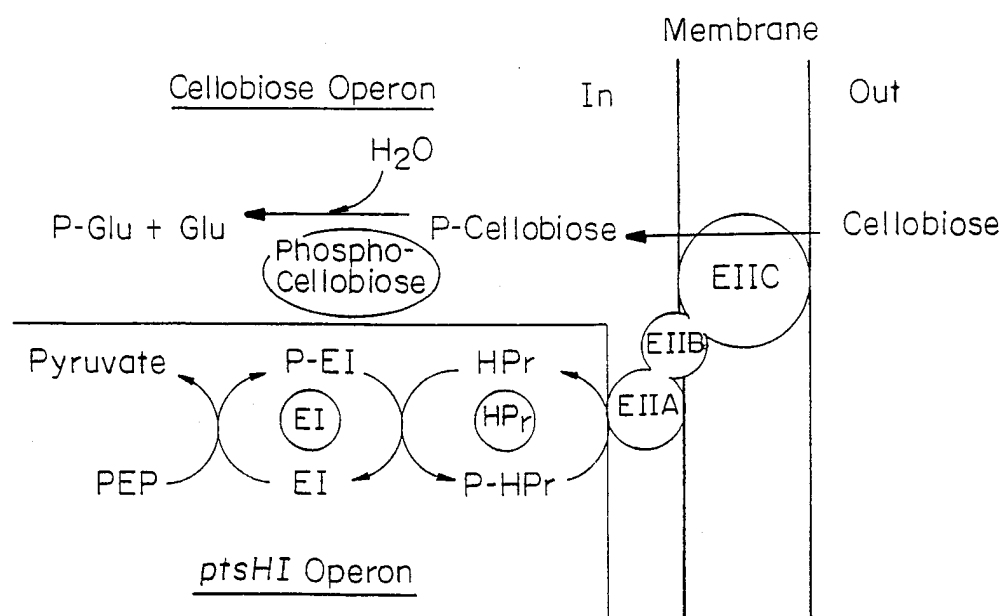


FIG. 13

INTERNATIONAL SEARCH REPORT

Inter. onal Application No

PCT/US 98/06331

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/54 C12N15/55 C12P7/10 C12N9/12 C12N9/42
C12N1/20 C12N15/01 C12P7/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	MONIRUZZAMAN M. ET AL.: "Isolation and molecular characterization of high-performance cellobiose-fermenting spontaneous mutants of ethanologenic Escherichia coli K011 containing the Klebsiella oxytoca casAB operon." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 63, no. 12, December 1997, pages 4633-4637, XP002071921 see abstract ---	1-32
Y	US 4 464 471 A (ARMENTROUT RICHARD W ET AL) 7 August 1984 see abstract see column 2, line 51 - line 61 --- -/--	1-32

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

Special categories of cited documents:

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- "O" document referring to an oral disclosure, use, exhibition or other means
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- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

17 July 1998

Date of mailing of the international search report

30/07/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Lejeune, R

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 98/06331

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LAI X. ET AL.: "Cloning of cellobiose phosphoenolpyruvate-dependent phosphotransferase genes: functional expression in recombinant Escherichia coli and identification of a putative binding region for disaccharides." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 63, no. 2, February 1997, pages 355-363, XP002071922 cited in the application see abstract see page 355, column 1, paragraph 1 ---	1-32
A	US 5 424 202 A (GUIMARAES WALTER V ET AL) 13 June 1995 cited in the application see abstract see column 62, paragraph 2 -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/06331

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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			AU 1017697 A	27-03-1997
			AU 672748 B	17-10-1996
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			WO 9216615 A	01-10-1992
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