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(54) Titre : SOUCHE UNIQUE DE ZYMOMONAS MOBILIS POUR FERMENTATION DE XYLOSE ET D'ARABINOSE
(54) Title: SINGLE ZYMOMONAS MOBILIS STRAIN FOR XYLOSE AND ARABINOSE FERMENTATION

(57) Abrégé/Abstract:
A microorganism of the genus Zymomonas containing exogenous genes encoding xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate 4-epimerase, transaldolase and transketolase and further comprising at least one promotor recognized by Zymomonas which regulates the expression of at least one of the genes, wherein the microorganism is capable of growing on arabinose and xylose as carbon sources and fermenting the arabinose and xylose to ethanol in about 75 % theoretical yield and, wherein the microorganism without the genes is incapable of growing on or fermenting the arabinose and xylose to ethanol.

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(54) Title: SINGLE *ZYMOMONAS MOBILIS* STRAIN FOR XYLOSE AND ARABINOSE FERMENTATION

(57) Abstract

A microorganism of the genus Zymomonas containing exogenous genes encoding xylose isomerase, xylulokinase, L–arabinose isomerase, L–ribulokinase, L–ribulose–5–phosphate 4–epimerase, transaldolase and transketolase and further comprising at least one promotor recognized by *Zymomonas* which regulates the expression of at least one of the genes, wherein the microorganism is capable of growing on arabinose and xylose as carbon sources and fermenting the arabinose and xylose to ethanol in about 75 % theoretical yield and, wherein the microorganism without the genes is incapable of growing on or fermenting the arabinose and xylose to ethanol.

Single Zymomonas Mobilis Strain for Xylose and Arabinose Fermentation

Contractual Origin of the Invention:

The United States Government has rights in this invention pursuant to Contract No. DE-AC36-83CH10093 between the United States Department of Energy and the Midwest Research Institute.

Field of the Invention:

This invention relates to a recombinant *Zymomonas mobilis* biocatalyst capable of converting xylose and arabinose along with glucose to ethanol.

This is a new, single *Zymomonas mobilis* biocatalyst capable of converting both xylose and arabinose along with glucose to ethanol. Seven genes (xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase-5-P 4-epimerase, transketolase, and transaldolase) which encode the enzymes necessary for converting xylose and arabinose to common intermediates of *Zymomonas'* central glycolic pathway were simultaneously introduced into *Zymomonas* under the control of strong promoters that direct their expression, even in the presence of glucose. The newly engineered strain can grow on and ferment either xylose, arabinose, or glucose as sole carbon sources to ethanol and ferments a mixture of 1% glucose, 2% xylose and 2% arabinose to ethanol at about 90% of the theoretical yield at 30°C without pH control.

Background Art:

Cellulosic biomass is a favorable feedstock for fuel ethanol production because it is both readily available and less expensive than either corn or sugarcane. However, substantial hurdles must be overcome before a typical cellulosic feedstock can be utilized

effectively as a substrate for the fermentative production of ethanol. The typical feedstock is comprised of approximately 35% - 45% cellulose, 30 - 40% hemicellulose, 15% lignin and 10% of other components. The cellulose fraction is comprised of polymers of the hexose sugar, glucose. The hemicellulose fraction is comprised mostly of pentose sugars and substantially of xylose.

Whereas microorganisms are known that can efficiently ferment the glucose component in cellulose, conversion of the xylose in the hemicellulose fraction to ethanol has been difficult and this remains to be one of the economical bottlenecks in the biomass to ethanol conversion scheme. The rapid and efficient utilization of the xylose component in cellulosic biomass is desirable in the development of a commercial process.

Zymomonas mobilis is a bacterium that has been utilized as a natural fermentative agent in the production of alcoholic beverages, such as pulque and palm wines produced from plant saps. Comparative performance trials have suggested that *Zymomonas* may become an important industrial ethanol-producing microorganism because of its 5 - 10% higher yield and up to 5-fold higher productivity compared to traditional yeast fermentations. Because of its potential value, several processes based on the use of Zymomonas for production of industrial ethanol from glucose-based feedstocks have been disclosed in U.S. Patent Nos. 4,731,329, March 1988 to Lawford, 4,812,410, March 1989 to Lawford, 4,816,399, March 1989 to Lawford and 4,876,196, October 1989 to Salzbrunn.

While *Zymomonas* may become an important fuel ethanol-producing microorganism from glucose-based feedstocks, its substrate utilization range is restricted to fermentation of glucose, sucrose and fructose and as such, it is not naturally suited for fermentation of the xylose component in cellulosic feedstocks. Zymomonas contains the Enter-Douderoff pathway that allows it to ferment glucose very efficiently to ethanol as the sole fermentation product. However, *Zymomonas* is naturally unable to ferment the xylose in cellulosic biomass because it lacks the essential pentose metabolism pathways. Thus, an opportunity exists to genetically engineer this organism for the fermentation of xylose to ethanol.

Genetic engineering attempts have been made to enhance ethanol production by fermentation by transferring genes from one species to another. For example, see U.S. Patent Nos. 5,000,000 and 5,028,539. Gene cloning and expression of various enzymes

including enzymes for creating a new metabolic pathway are also known. For example see U.S. Patent Nos. 5,272,073; 5,041,378; 5,168,056 and 5,226,475. However, none of these discoveries has successfully broadened the fermentable substrate range of a microorganism which could not previously ferment xylose to ethanol.

Previous attempts to introduce a xylose catabolic pathway from other Xanthomonas or Klebsiella into Zymomonas have been unsuccessful and the recombinant strains were incapable of growth on xylose as the sole carbon source (Feldmann et al, 1992, Appl. Microbiol. Biotechnol. 38:354 - 361; Liu et al, 1988. J. Biotechnol. 7:61 - 77).

The wild-type *Z. mobilis* ferments only glucose, sucrose and fructose and is not able to utilize pentoses, such as D-xylose and L-arabinose. Enzymatic analysis indicated that *Z. mobilis* lacks functional pentose metabolic pathways necessary to ferment D-xylose and L-arabinose. We have developed the xylose-fermenting *Z. mobilis* by introduction and expression of four genes encoding D-xylose-assimilating enzymes, xylose isomerase and xylulokinase as well as pentose-phosphate pathway enzymes, transaldolase and transketolase (Picataggio et al, U.S. 5,514,583, May 7, 1996). By introducing the genes encoding xylose isomerase and xylulokinase, xylose can be converted to xylulose 5 P. Then, by introducing two more genes encoding enzymes in the pentose phosphate pathway, comprising transaldolase and transketolase, xylulose-5-P can be further converted to the key intermediates that couple pentose metabolism to the glycolytic Entner-Douderoff pathway and consequently, to ethanol production. Independently, we have also developed the arabinose-fermenting *Z. mobilis* by introduction of five genes encoding L-arabinose-assimilating enzymes, L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase as well as the pentose-phosphate pathway enzymes, transaldolase and transketolase (Canadian Patent File No. 2,173,793, Picataggio et al laid open October 15, 1966). By introducing the genes encoding L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate-4-epimerase, arabinose can be converted to xylulose-5-P. Similarly, by introducing the genes encoding enzymes in the pentose phosphate pathway, comprising transaldolase and transketolase, xylulose-5-P can be further converted to the key intermediates that couple pentose metabolism to the glycolytic Entner-Douderoff pathway and consequently, to ethanol production.

Disclosure of the Invention:

An aspect of the present invention provides a microorganism of the genus *Zymomonas* that expresses exogenous genes encoding xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L- ribulose-5-phosphate 4-epimerase, transaldolase and transketolase and further comprising at least one promoter recognized by *Zymomonas*, wherein said microorganism is capable of growing on arabinose and xylose.

A further aspect provides for a vector comprising genes encoding xylose isomerase, xylulokinase, L- arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate 4-epimerase, transaldolase and transketolase and at least one promoter recognized by *Zymomonas* that regulates expression of the genes.

The present invention provides a single *Zymomonas mobilis* biocatalyst capable of converting both xylose and arabinose along with glucose to ethanol. Seven genes (xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase- 5-P 4-epimerase, transketolase and transaldolase) which encode the enzymes necessary for converting xylose and arabinose to common intermediates of *Zymomonas*' central glycolytic pathway were simultaneously introduced into *Zymomonas* under the control of strong promoters that direct their expression, even in the presence of glucose. The newly engineered strain can grow on and ferment either xylose, arabinose, or glucose as sole carbon sources to ethanol and ferments a mixture of 1% glucose, 2% xylose and 2% arabinose to ethanol at about 90% of the theoretical yield at 30°C without pH control.

A further aspect of the present invention is to enable the *Z. mobilis* to ferment both xylose and arabinose, by introducing and expressing the genes for both xylose-assimilating enzymes, xylose isomerase and xylulokinase and L-arabinose-assimilating enzymes, L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, as well as the pentose-phosphate pathway enzymes, transaldolase and transketolase into *Z. mobilis*. The cloned genes may be provided on any number of vectors but preferably are contained on a single plasmid vector. More preferably, the genes are integrated into the host genome.

The material source for the genes encoding pentose metabolism is selected from the group consisting of: Xanthomonas, Klebsiella, *E. coli*, Rhodobacter, Flavobacterium, Acetobacter, Gluconobacter, Rhizobium, Agrobacterium, Salmonella and Pseudomonads. A preferred organism for the source of genes is *E. coli*. The preferred genes encode xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, transaldolase and transketolase.

Another aspect of the present invention is cultures of microorganisms with the above-described abilities. The cultures may be biologically pure or be mixed with other strains of different organisms to aid in the metabolism of the substrates or a mixture of substrates into ethanol.

Yet another aspect of the invention is a process for producing a newly

engineered strain that can grow on and ferment either xylose, arabinose or glucose as sole carbon sources to ethanol.

An additional aspect of the invention is to provide a new *Zymomonas* strain that will be useful for those lignocellulosic feedstocks containing significant quantities of both the pentose sugars (such as switch grass, wheat straw, corn cobs, corn fiber and spent grains).

Brief Description of the Drawings:

Figure 1 shows a schematic of a process of enabling *Z. mobilis* to ferment both xylose and arabinose by introducing and expressing the genes from both xylose-assimilating enzymes, xylose isomerase and xylulokinase and L-arabinose-assimilating enzymes, L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase, as well as the pentose-phosphate pathway enzymes, transaldolase and transketolase into *Z. mobilis*.

Figure 2, (composed of Figures 2a - 2d), depicts the plasmid maps pZB301, pZB401, pZB402 and pZB403.

Figure 3 shows the ethanol yields using a control *Zymomonas mobilis* and the present recombinant strain when grown on certain sugars or a mixture of certain sugars as the carbon source.

Description of Preferred Embodiments:

The invention is the preparation of plasmids of all seven genes, xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate 4-epimerase, transaldolase and transketolase under control of strong, constitutive *Z. mobilis* promoters that allow high expression of all the genes and transformed *Z. mobilis*. Specifically, xylose isomerase and xylulokinase are controlled under the *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter in a P_{gap}-xylAxylB operon; L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase are also controlled under the *Z. mobilis* GAP promotor in a P_{gap}-araBAD operon; transaldolase and transketolase are controlled under the *Z. mobilis* enolase (ENO) promoter in a P_{eno}-tal/

tkt operon.

The genes encoding xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase and L-ribulose 5-phosphate 4-epimerase, transaldolase and transketolase may be obtained from bacteria selected from the group consisting of Xanthomonas, Klebsiella, E. coli, Rhodobacter, Flavobacterium, Acetobacter, Gluconobacter, Rhizobium, Agrobacterium, Salmonella and Pseudomonads, preferably E. coli.

Example I

Selection of Appropriate Z. mobilis Hosts for Transformation and Expression of Arabinose and Xylose Utilization Genes

While the wild-type strain of *Z. mobilis* 39676 transformed with pZB4L carrying the genes for four xylose metabolism enzymes acquired Xyl^+ phenotype, transformation of pZB206 into the wild-type *Z. mobilis* 39676 did not provide the ability to grow on L-arabinose containing media. It was apparent that mutation event(s) required for conferring Ara+ in addition of expressing ara genes in *Z. mobilis* might have occurred on the chromosomal DNA of the arabinose-fermenting *Z. mobilis* 39676(pZB206) strain when constructed. Therefore, efforts were made to cure pZB206 from the strain 39676 (pZB206) to obtain an appropriate host strain by culturing the strain in a non-selective medium (i.e. without tetracycline (Tc)) at 37°C for about 20 generations. The cells lost from the plasmid were confirmed by testing its Tc sensitivity and Xyl phenotype through replica plating as well as by mini-screening of the plasmid. The pZB206 cured strain was designed *Z. mobilis* 206°C. Transformation of this strain individually with pZB206 (arabinose plasmid) (See Canadian patent File No. 2,173,793) pZB4L (xylose plasmid) (Picataggio et al, U.S. 5,514,583) and pZB4L (xylose plasmid) separately conferred Ara+ and Xyl^+ phenotypes to the corresponding transformants. These results indicated the *Z. mobilis* 20°C carried mutation(s) contributing to Ara+ phenotypes. Therefore, *Z. mobilis* 206°C was chosen to be the host for expressing both arabinose and xylose utilization genes.

Example II

Expression of Xylose and Arabinose Utilization Genes in *Z. mobilis* 206°C

Plasmids were constructed containing all seven genes, xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate 4-epimerase,

transaldolase and transketolase under control of strong, constitutive Z. *mobilis* promotors that allow high expression of all the genes and transformed Z. *mobilis*, Specifically, xylose isomerase and xylulokinase are controlled under the Z. *mobilis* glyceraldehyde-3-phosphate dehydrogenase (GAP) promotor in a P_{gap}-xylAxylB operon; L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase are also controlled under the Z. *mobilis* GAP promotor in a P_{gap}-araBAD operon; transaldolase and transketolase are controlled under the Z. *mobilis* enolase (ENO) promotor in a P_{eno}-tal/tkt operon.

Example III

Development Of A Single Strain With Both Xylose And Arabinose-Fermentating Capability

To develop a single strain with both xylose and arabinose-fermentating capability a single plasmid was constructed containing xylose and arabinose utilization genes which includes xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate-4-epimerase, transaldolase, and transketolase. The xylose plasmid pZB4 was partially digested with NotI followed by ligation with a gel purified 4.4-kb NotI fragment containing P_{gap}-araBAD operon from pZB206. The ligation mixture was used to electroporate E. *cold* B/r araA - and cells were plated on MacConkey plates containing L-arabinose and Tc.

The $Ara^{+}Tc^{r}$ transformants were examined for the presence of the correct P_{gap}-araBAD operon insert in pZB4. DNA analysis of transformants indicated that NotI insert containing P_{gap}-araBAD operon was inserted clockwise in the NotI site upstream to P_{gap}-xyl/AxylB operon. The resulting plasmid, designated as pZB301 (Fig. 2), was then used to electroporate Z. *mobilis*. Z. . mobilis 206C transformed with pZB301 was able to grow on media containing either L-arabinose or D-xylose as sole carbon source in the presence of Tc.

Example IV

Preparation of a Single Plasmid containing Xylose and Arabinose Utilization Genes Based on Another Xylose Plasmid pZBL

Efforts were made to construct a single plasmid containing xylose and arabinose utilization genes based on another xylose plasmid pZB4L. Similarly, pZB4L was digested with NOTI followed by ligation with a gel purified 4.4-kb NotI fragment containing P_{gap}-araBAD operon from PZB206. *E. Coli* HB101 (instead of B/r ara A-) was electroporated with the ligation mixture followed by selection on MacConkey+ara+Tc plates. The $Ara^{+}Tc^{r}$ transformants were examined for the presence of the correct P_{gap}-araBAD operon insert in pZB4L. DNA analysis indicated three types of orientations of the insert. These plasmids were designated as pZB401, pZB402, and pZB403 (Fig. 2), and were then used to electroporate 8. *mobilis* 206C. Z. *mobilis* 206C transformed with pZB401, pZB402 or pZB403 and were able to grow on media containing either L-arabinose or D-xylose as a sole carbon source in the presence of To.

Enzymatic analysis confirmed that all seven enzymes are highly expressed as compared to the control strain containing vector pZB186 (Table 1).

Table 1. Summary of Enzymatic Assays

Strains[1]	Specific Enzyme Activity (U/Mg protein)						
	X1	XK	L-AI	L-RK	L-Repi	TKT	TAL
206C(pZB186)	0.03	0.08	nd[2]	0.58	0.01	0.01	0.07
206C(pZB301)	0.29	3.30	3.25	1.34	0.22	0.55	2.04
206C(pZB401)	0.25	0.82	2.22	1.56	0.29	0.57	2.25

[1]Cells were collected at OD_{600}=1.2.

[2]nd=not detected

Fermentation Performance of the Combined Xylose and Arabinose-Fermenting Z. *mobilis* strains

To investigate the fermentation performance of these newly constructed combined xylose and arabinose-fermenting Z. *mobilis* strains, fermentations were performed statically at 30°C without pH control in bottles with 80 ml RM+Tc containing mixtures of 2% arabinose +2% xylose or 1% glucose +2% arabinose +2% xylose for strains of 206C(pZB301), 206C(pZB401), 206C(pZB402) and 206C (pZB403) .

In the absence of glucose, all four strains fermented a mixture of 2% xylose and 2% arabinose at 63-66% of ethanol yields (process) in about 48 hours. The ethanol yields based on consumed sugars ranged from 0.45 to 0.48 g/g. The low process yields resulted from residual arabinose, apparently due to a decrease in the pH during fermentation without pH control. Although it is not clear whether both arabinose and xylose were transported into the cell by the same sugar transport system it appears that the presence of the arabinose does not inhibit xylose utilization and vice versa. The sugars are simultaneously utilized. However, the rate of the utilization is much higher in xylose as compared with arabinose. A representative fermentation profile is shown in Figure 3.

In the presence of 1% glucose, all four strains fermented a mixture of 2% xylose and 2% arabinose at 72-75% of ethanol yields (process) in about 48 hours. The ethanol yields based on consumed sugars ranged from 0.44 to 0.48 g/g. Glucose is preferentially utilized at a faster rate than xylose and arabinose. The growth started earlier and reached about 1.6-2 fold higher OD for all strains than in the absence of glucose. Apparently, addition of the glucose facilitated pentose utilization and resulted in higher process yields.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A microorganism of the genus *Zymomonas* that expresses exogenous genes encoding xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L- ribulose-5-phosphate 4-epimerase, transaldolase and transketolase and further comprising at least one promoter recognized by *Zymomonas*, wherein said microorganism is capable of growing on arabinose and xylose.

2. A microorganism according to claim 1, wherein the genes encoding xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose 5-phosphate 4-epimerase, transaldolase and transketolase were obtained from bacteria selected from the group consisting of Xanthomonas, Klebsiella, *E. coli*, Rhodobacter, Flavobacterium, Acetobacter, Gluconobacter, Rhizobium, Agrobacterium, Salmonella and Pseudomonads.

3. A microorganism according to claim 1, wherein the genes are integrated into the genome of the microorganism.

4. A microorganism according to claim 1, wherein said genes are contained on a vector.

5. A microorganism according to claim 1, wherein the at least one promoter is the *Z.mobilis* glyceraldehyde-3-phosphate dehydrogenase promoter in a (P_{gap}) or the *Z.mobilis* enolase (P_{eno}) promoter.

6. A microorganism according to claim 5, wherein said genes encoding xylose isomerase and xylulokinase are expressed under the control of a glyceraldehyde-3-phosphate dehydrogenase promoter in a P_{gap}-xylAxylB operon recognized by *Zymomonas*; the genes encoding L-arabinose isomerase, L-ribulokinase and

L-ribulose-5-phosphate 4-epimerase are expressed under the control of a glyceraldehyde-3-phosphate dehydrogenase promoter in a P_{gap}-araBAD operon recognized by *Zymomonas;* and the genes encoding transaldolase and transketolase are expressed under the control of an enolase promoter in a P_{eno}-tal/tkt operon recognized by *Zymomonas*.

7. A vector comprising genes encoding xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate 4-epimerase, transaldolase and transketolase and at least one promoter recognized by *Zymomonas* that regulates expression of said genes.

8. A vector according to claim 7, wherein the at least one promoter is the *Z.mobilis* glyceraldehyde-3-phosphate dehydrogenase promoter (P_{gap}) or the *Z.mobilis* enolase (P_{eno}) promoter.

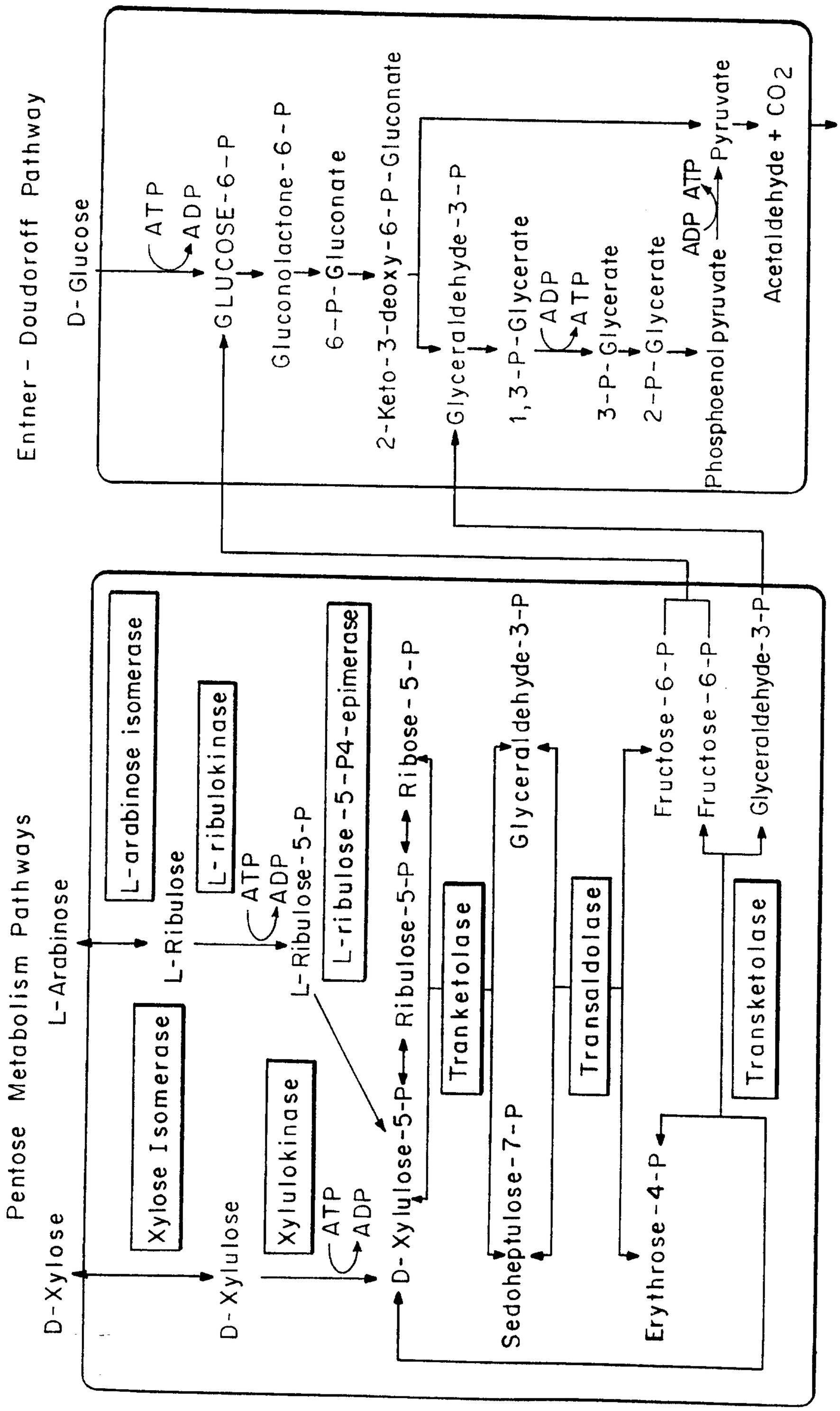
9. A vector according to claim 8, wherein the genes encoding xylose isomerase and xylulokinase are regulated by a glyceraldehyde-3-phosphate dehydrogenase promotor in a P_{gap}-xylAxylB operon recognized by *Zymomonas*.

10. A vector according to claim 7, wherein the genes encoding L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate 4-epimerase are regulated by a glyceraldehyde-3-phosphate dehydrogenase promoter P_{gap}-araBAD operon recognized by *Zymomonas*.

11. A vector according to claim 7, wherein the genes encoding transaldolase and transketolase are regulated by an enolase promoter in a P_{eno}-tal/tkt operon recognized by *Zymomonas*.

12. A microorganism according to claim 2, wherein the genes encoding xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate-4-epimerase, transaldolase and transketolase are obtained from *E. coli*.

FIG. 1


Metabolism Pathways
L-Arabinose
L-arabinose isomerase
L-Ribulose
L-ribulokinase
ATP
ADP
L-Ribulose-5-P
L-ribulose-5-P4-epimerase
Ribulose-5-P ↔ Ribose-5-P
Tranketolase
Glyceraldehyde-3-P
Transaldolase
Fructose-6-P
Fructose-6-P
Glyceraldehyde-3-P
Transketolase
Sedoheptulose-7-P
Erythrose-4-P
Pentose Metabolism
D-Xylose
Xylose Isomerase
D-Xylulose
Xylulokinase
ATP
ADP
D-Xylulose-5-P
Entner - Doudoroff Pathway
D-Glucose
ATP
ADP
GLUCOSE-6-P
Gluconolactone-6-P
6-P-Gluconate
2-Keto-3-deoxy-6-P-Gluconate
Glyceraldehyde-3-P
1,3-P-Glycerate
ADP
ATP
3-P-Glycerate
2-P-Glycerate
ADP ATP
Phosphoenol pyruvate
Pyruvate
+ CO2
Acetaldehyde +
Ethanol

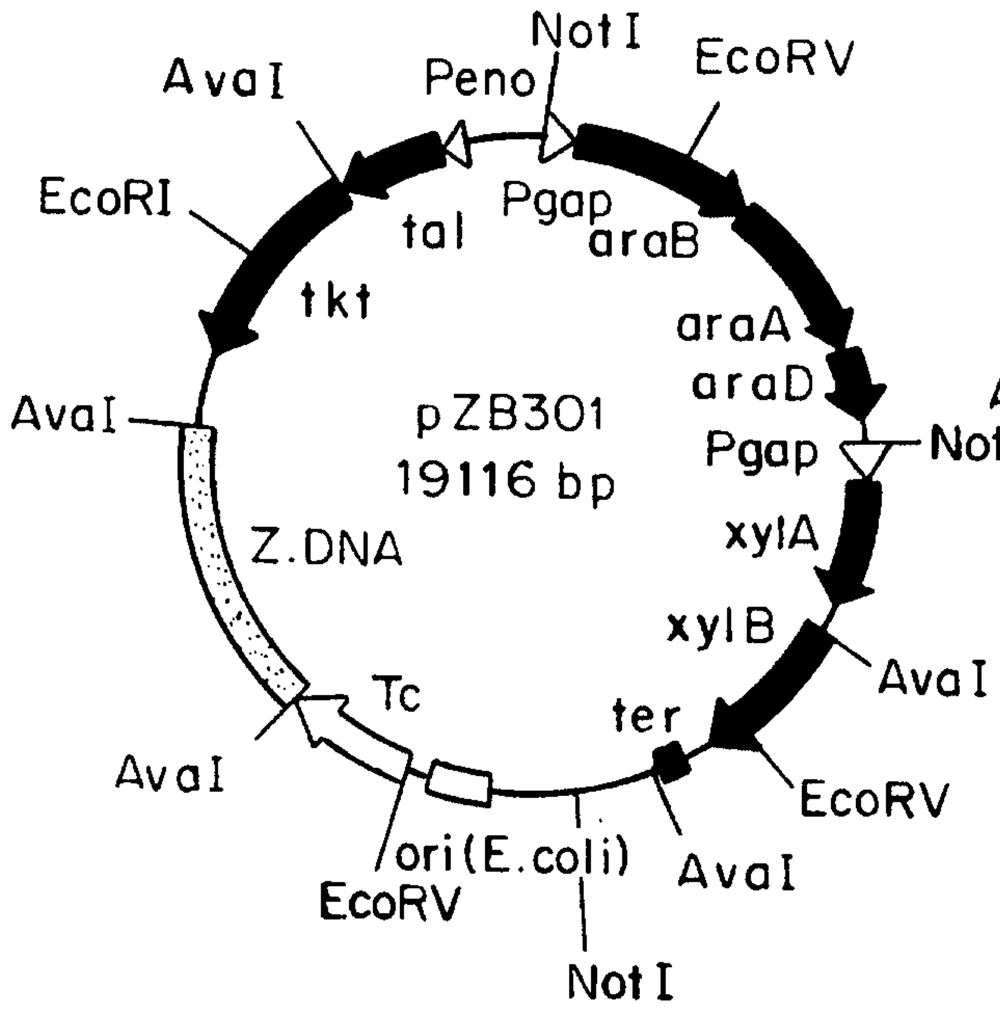


FIG. 2a

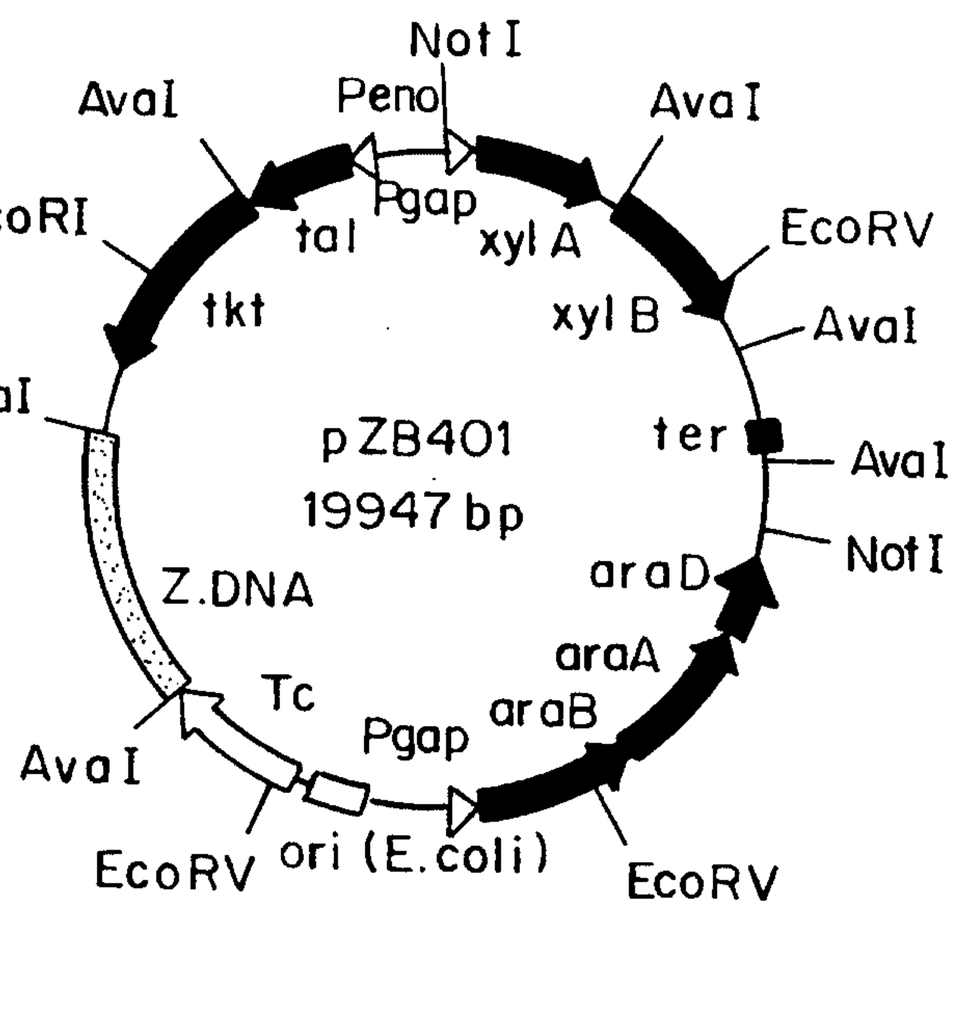


FIG. 2b

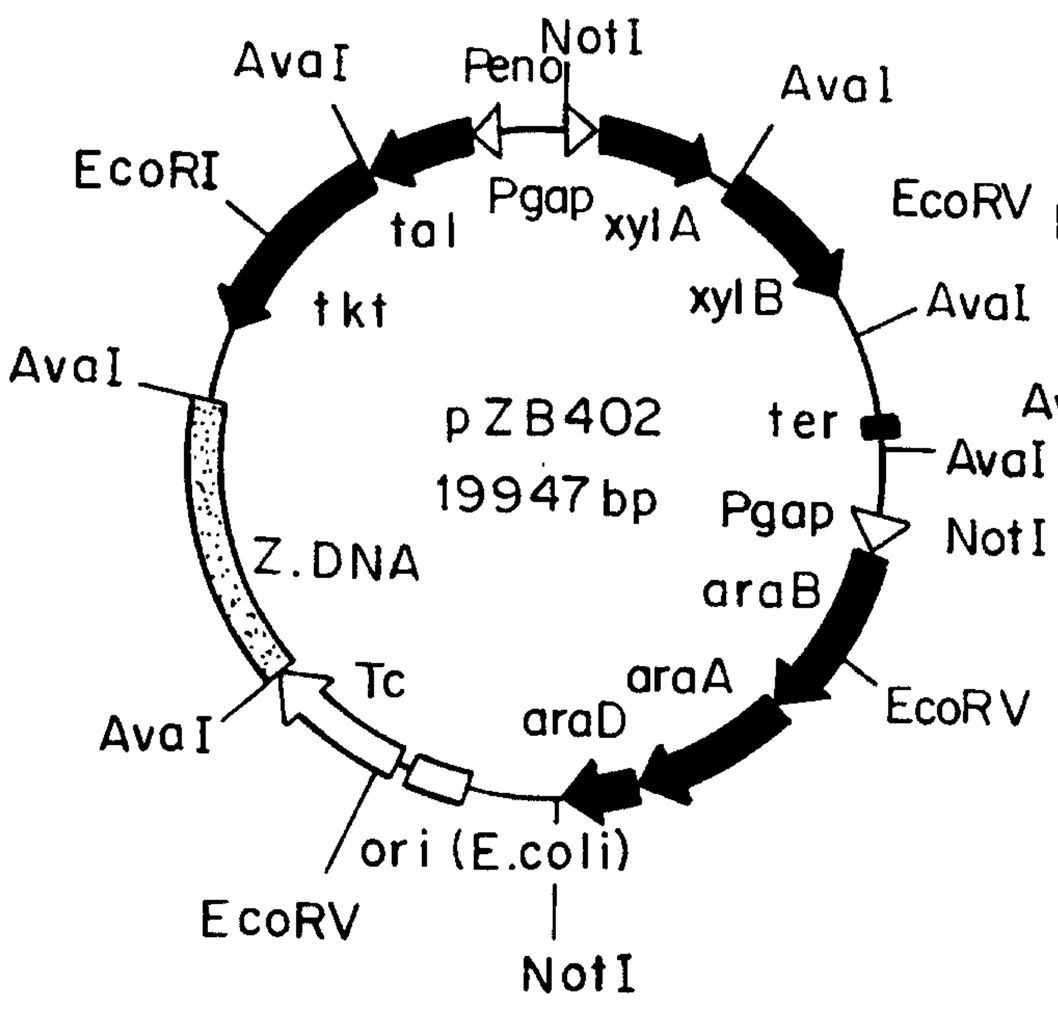


FIG. 2c

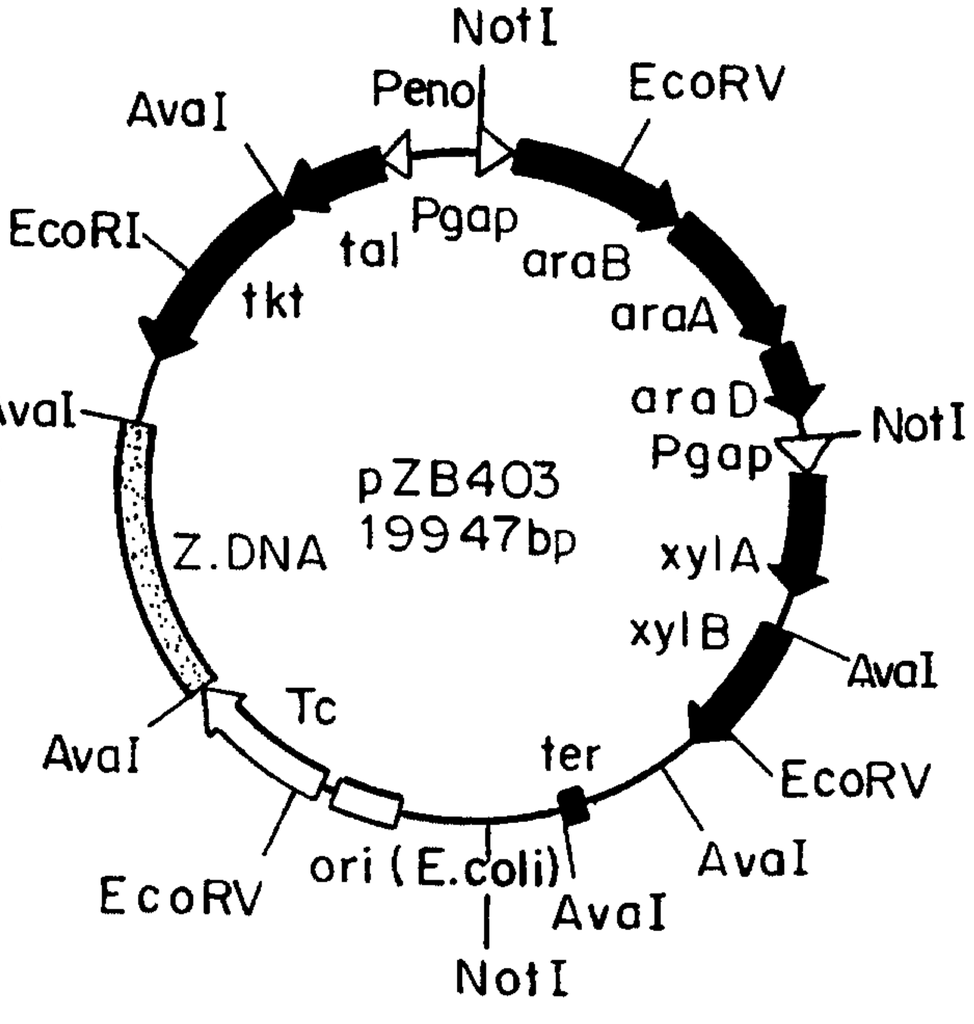
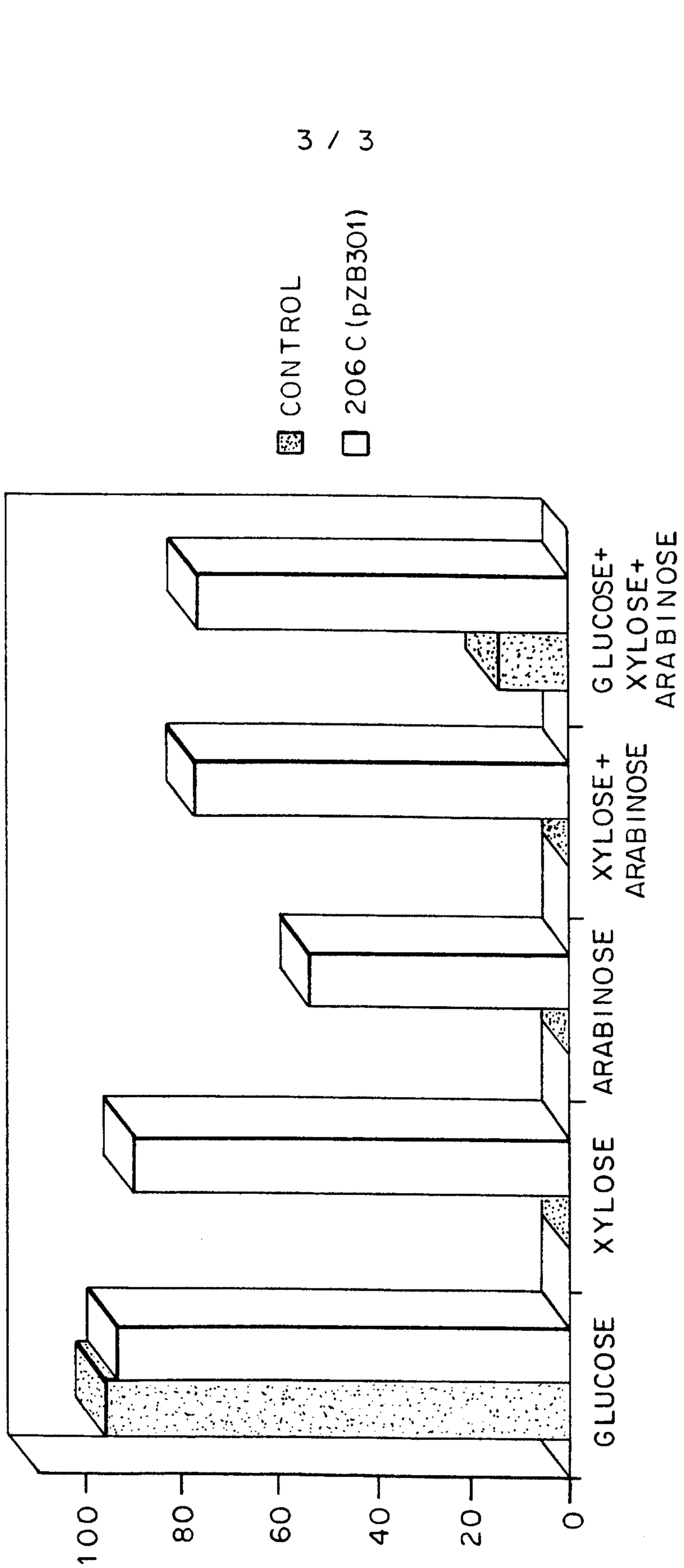


FIG. 2d

FIG. 3


CONTROL
206 (pZB301)
GLUCOSE+ XYLOSE+ ARABINOSE
XYLOSE+ ARABINOSE
XYLOSE ARABINOSE
GLUCOSE
100
80
60
40
20
0
% ETHANOL YIELD (PROCESS)