



(22) Date de dépôt/Filing Date: 2000/05/02

(41) Mise à la disp. pub./Open to Public Insp.: 2001/11/02

(51) Cl.Int.⁷/Int.Cl.⁷ C12N 1/21, C12N 15/63, C12N 15/11,
C12P 7/04

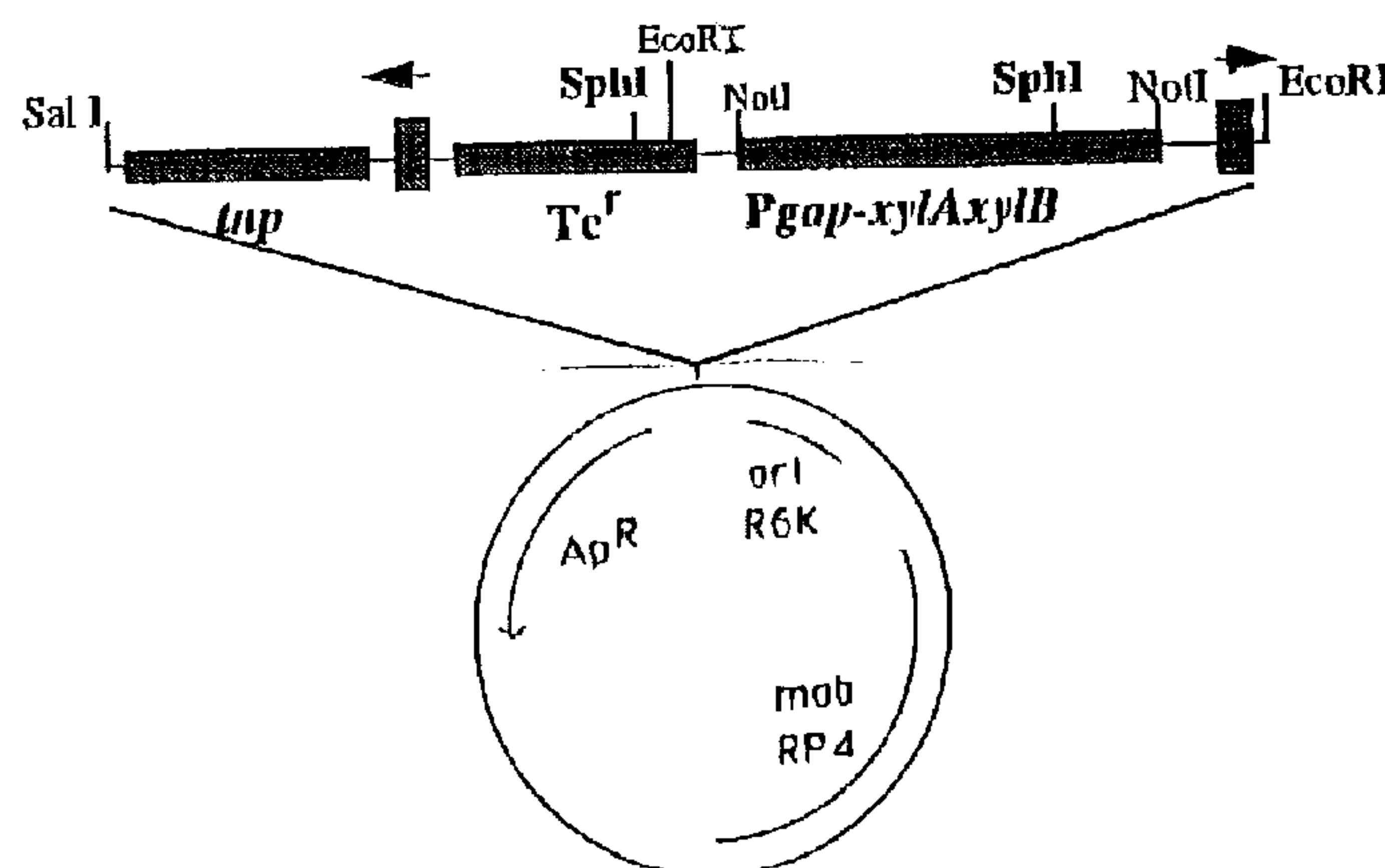
(71) Demandeur/Applicant:
MIDWEST RESEARCH INSTITUTE, US

(72) Inventeurs/Inventors:
CHOU, YAT-CHEN, TW;
ZHANG, MIN, US

(74) Agent: FINLAYSON & SINGLEHURST

(54) Titre : SOUCHES STABLES DE ZYMOMONAS MOBILIS POUR LA FERMENTATION DU XYLOSE ET DE
L'ARABINOSE

(54) Title: STABLE ZYMOMONAS MOBILIS XYLOSE AND ARABINOSE FERMENTING STRAINS



Mini-Tn5 Tc with Xylose Assimilation Operon.

(57) **Abrégé/Abstract:**

The present invention briefly includes a transposon for stable insertion of foreign genes into a bacterial genome, comprising at least one operon having structural genes encoding enzymes selected from the group consisting of xylA xylB, araBAD and tal/tkt, and at least one promoter for expression of the structural genes in the bacterium, a pair of inverted insertion sequences, the operons contained inside the insertion sequences, and a transposase gene located outside of the insertion sequences. A plasmid shuttle vector for transformation of foreign genes into a bacterial genome, comprising at least one operon having structural genes encoding enzymes selected from the group consisting of xylA xylB, araBAD and tal/tkt, at least one promoter for expression of the structural genes in the bacterium, and at least two DNA fragments having homology with a gene in the bacterial genome to be transformed, is also provided. The transposon and shuttle vectors are useful in constructing significantly different Zymomonas mobilis strains, according to the present invention, which are useful in the conversion of the cellulose derived pentose sugars into fuels and chemicals, using traditional fermentation technology, because they are stable for expression in a non-selection medium.

STABLE ZYMOMONAS MOBILIS XYLOSE AND ARABINOSE FERMENTING STRAINS

ABSTRACT

The present invention briefly includes a transposon for stable insertion of foreign genes into a bacterial genome, comprising at least one operon having structural genes encoding enzymes selected from the group consisting of *xylA**xylB*, *araBAD* and *tal/iki*, and at least one promoter for expression
5 of the structural genes in the bacterium, a pair of inverted insertion sequences, the operons contained inside the insertion sequences, and a transposase gene located outside of the insertion sequences. A plasmid shuttle vector for transformation of foreign genes into a bacterial genome, comprising at least one operon having structural genes encoding enzymes selected from the group consisting of *xylA**xylB*, *araBAD* and *tal/iki*, at least one promoter for expression of the structural genes in the bacterium, and
10 at least two DNA fragments having homology with a gene in the bacterial genome to be transformed, is also provided.

The transposon and shuttle vectors are useful in constructing significantly different *Zymomonas mobilis* strains, according to the present invention, which are useful in the conversion of the cellulose derived pentose sugars into fuels and chemicals, using traditional fermentation
15 technology, because they are stable for expression in a non-selection medium.

STABLE ZYMOMONAS MOBILIS XYLOSE AND ARABINOSE FERMENTING STRAINS

INVENTORS: Min Zhang, Lakewood, CO; and Yat-Chen Chou, Taipei, Republic of
5 Taiwan.

BACKGROUND OF THE INVENTION

1. Field of the Invention.

The present invention relates to the biological conversion of cellulosic substrates into fuels and chemicals, and in particular to recombinant *Zymomonas mobilis* strains which ferment xylose and
10 arabinose, or both, into ethanol.

2. Description of the Related Art.

Fermentation technology is useful for the conversion renewable biomass cellulose substrates into fuels and chemicals, such as ethanol. A typical substrate is comprised of 35-45% cellulose, 30-40% hemicellulose, and 15% lignin. The hydrolysis fraction contains glucose polymers, and the
15 hemicellulose fraction contains mostly xylose. Arabinose is also a significant fermentable substrate found in biomass materials, such as switchgrass grass and corn fiber. Thus, achieving a high rate of specific product formation and conversion efficiency in the fermentation of the pentose sugars is vital to the commercial production of fuels and chemicals from a renewable substrates.

Z. mobilis is widely reported for its ability to rapidly and efficiently convert glucose substrates
20 into ethanol, at a low pH, in an anaerobic culture, and in a medium which contains the inhibitory compounds typically associated with a lignocellulose-hydrolysate. A distinct disadvantage in the use of *Z. mobilis* is, however, that it does not ferment pentose sugars. To overcome this disadvantage, the prior art has focused on recombinant *Z. mobilis* strains which ferment a mixture of glucose, and xylose or arabinose, or both, using exogenous genes which catalyze the metabolism of xylose and
25 arabinose. These strains, and the cloning vectors, are based on the use of multiple-copy plasmids having antibiotic resistance markers.

US Pat. No. 5,514,583 discloses a transformed *Z. mobilis* xylose fermenting strain (CP4/pZB4 and pZB5) having exogenous genes, and plasmid vectors (pZB4 and pZB5) encoding xylose isomerase, xylulokinase, transaldolase and transketolase, and further comprising at least one

promoter (*Pgap and Peno*) recognized by *Zymomonas* which regulates the expression of at least one of said genes. The microorganism is capable of growing on xylose as a sole carbon source, and fermenting xylose to ethanol at about 88% of the maximum theoretic yield. The Patent claims an integrated strain.

5 US Pat. Nos. 5,712,133 and 5,726,053 disclose, *inter alia*, *Z. mobilis* arabinose fermenting transformants (39676/pZB 206), containing exogenous genes that encode L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate-4-epimerase, transaldolase and transketolase which impart arabinose to ethanol fermentation capability. The plasmid vector (pZB 206) and a process of using the transformants for the fermentation of a glucose and arabinose containing substrate is also
10 disclosed. The Patent claims integration of the exogenous genes into the host genome.

US Pat. No. 5,843,760 discloses a *Z. mobilis* xylose and arabinose fermenting transformant (206C/pZB301) 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 promoter recognized by *Zymomonas* which regulates the expression of
15 at least one of said genes, wherein said microorganism is capable of growing on arabinose and/or xylose, alone or in combination, as the carbon source and fermenting said arabinose and xylose to ethanol. The process of using the transformants together with the plasmid vectors (pZB301, pZB401, pZB402, and pZB 403) is also disclosed. This Patent claims integration of the exogenous genes into the host genome.

20 Although hybrid plasmids may be readily maintained in *Z. mobilis* when cultivated in monoculture under controlled conditions, they frequently become unstable when the host organism is grown in the absence of selection pressure for plasmid maintenance, i.e., in the presence of antibiotics. For example, the exogenous genes in the above referenced strains are capable of stable expression for about forty generations. Instability may be exacerbated when *Z. mobilis* has to compete with other
25 organisms in a mixed culture, such as a cellulose simultaneous-saccharification-fermentation process. In addition, antibiotic resistance markers are generally perceived as undesirable for industrial application, such as the large-scale production of ethanol. Thus, it is preferable to insert the cloned genes into the *Z. mobilis* genome, where they are maintained at a low, natural copy number, and are thus not over-expressed, and where, at least theoretically, they should be as stable as genomic DNA.

In *Escherichia coli*, the classical method for generating genomic inserts of foreign genes involves the use of specialized λ phage cloning vectors that can exist stable in the lysogenic state. Alternatively, genes can be inserted through homologous recombination, when bracketed with *E. coli* chromosomal sequences, or by transposition if the genes can be cloned in the permissive sites of a transposon. While transposition has been demonstrated in *Z. mobilis*, Pappas, K. M., *et al.*, (1997) Transposon mutagenesis and strain construction in *Zymomonas mobilis*, *Journal of Applied Microbiology*, Vol. 82, p.p. 379-388 it has been limited to mini $M\mu$ or Tn5 multiple transposition of random auxotrophy or antibiotic resistance phenotypes for genetic analysis, and in the case of the Tn5 derivatives the insertion are reportedly stable for only 5-15 generations. Pappas, K. M., *et seq.* P. 383, FIG. 1. Moreover, site-specific insertion through homologous recombination in *Z. mobilis* has not been demonstrated, and no bacteriophage has ever been isolated from *Zymomonas*.

Transposons Tn5 and Tn10 are well known and have been widely used for mutagenesis and insertion of cloned DNA into a variety of gram-negative bacteria. In Herrero, M., *et al.*, (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* 172:6557-6567, a procedure is disclosed for cloning and stable insertion of foreign genes into the chromosome of gram-negative eubacteria by combining two sets of plasmids, (i) the transposition features of Tn10 and Tn5, (ii) the resistance to certain compounds, and (iii) the suicide delivery properties of the R6K-based plasmid pGP704. The resulting constructions contain unique *NotI* or *SfiI* sites internal to either the Tn10 or the Tn5 inverted repeats. These sites are used for cloning DNA fragments with the help of two additional specialized cloning plasmids, pUC18Not and pUC18Sfi. The newly derived constructions are maintained only in donor host strains that produce the R6K-specified π protein, which is an essential replication protein for R6K and plasmids derived therefrom. Donor plasmids containing hybrid transposons are transformed into a specialized λ pir lysogenic *E. coli* strain, such as *E. coli* Sm10(λ pir), with a chromosomally integrated RP4 that provided broad-host range conjugal transfer functions. Delivery of the donor plasmids into selected host bacteria is accomplished through mating with the target strain. Transposition of the hybrid transposon from the delivered suicide plasmid to a replicon in the target is mediated by the cognate transposase encoded on the plasmid at a site external to the transposon. Since the transposase function is not maintained in target cell, such

cells are immune to further transposition rounds. Multiple insertions in the same strain are therefore only limited by the availability of distinct selection markers.

Further disclosed in Herrero, M. *et al.*, 1990, Mini-Tn5 Transposon Derivatives for Insertion Mutagenesis Promoter Probing, and Genomic Insertion of Cloned DNA in Gram-Negative Eubacteria, *Journal of Bacteriol.* Vol 172, No. 11, p. 6568-6572, is the construction of a collection of Tn5-derived minitransposons, such as Tn5Tc, that enables the introduction of foreign DNA fragments into the chromosome of a variety of gram-negative bacteria. The minitransposons consist of genes specifying the resistance to kanamycin, and tetracycline as selection markers and a unique *NotI* cloning site flanked by 19-base-pair terminal repeat sequences of Tn5. The transposons are located on a R6K-based suicide delivery plasmid that provides the IS50_R transposase *tnp* gene in *cis* but external to the mobile element and whose conjugal transfer to recipients is mediated by RP4 mobilization functions in the donor. Therefore, insertions produced by these elements are generally more stable because of the absence of transposase-mediated secondary transpositions, deletions, and inversions. See also, Berg *et al.*, (1989) Transposable elements and the genetic engineering of bacteria, p.p. 879-926, in D.E. Berg, *Mobile DNA*, American Society for Microbiology, Washington, DC. Very stable insertions can in this way be obtained with elements derived, for instance also from Tn10. Way, J.C. *et al.*, (1984) New Tn10 derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. *Gene* 32: 369-379.

The structure of mini-Tn5Tc, Herrero, *et seq.*, p. 6569, is disclosed for use for insertion mutagenesis or as a transposon vector for the cloning of DNA fragments flanked by *NotI* sites (readily isolated by cloning DNA fragments first into the pUC18 derivatives pUC18Not and pUC18Not). The Mini-Tn5Tc element is constructed, *in vitro*, using standard recombinant DNA techniques. Maniatis, T., *et al.*, (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. The determinant for tetracycline (Tc) resistance is obtained as an *EcoRI* fragment from plasmids bearing them as an interposon. Fellay, R., *et al.* (1987) Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertion mutagenesis of Gram-negative bacteria. *Gene* 52: 147-154. This fragments is subsequently cloned into the single *EcoRI* site of pUC18Sfi, excised as an *SfiI* fragment, and inserted between the Tn5 19-base pair termini in pUT so that the mobile unit is present in all cases as an *XbaI-EcoRI* (partial) portion of the delivery plasmid. The resulting element is mini-Tn5Tc.

Tn10-based transposon vector delivery systems are described in Herrero, M., *et seq.* 172:6557-6567. Phage λ , a derivative λ RP167, carries a 5.1-kb *EcoRI* insert containing the mini-Tn10Km element and the transposase gene of IS10_R is located outside the inverted repeats of the mobile element and downstream of the *pTac* promoter. To obtain a transposon delivery plasmid with
 5 a host-independent regulation of its transposition, the *EcoRI* insert fragment is ligated to pBOR8, a derivative of pGP704 containing the *lacI^P* gene from plasmid pMJR1560. This plasmid is unable to replicate in host strains devoid of the R6K-specified π protein product of the *pir* gene. pGP704 contains the conjugal transfer origin (*oriT* sequence) of the RP4 plasmid and can therefore be transferred to gram-negative bacteria when provided in *trans* with mobilization (Mob) functions. The
 10 *MluI* fragment internal to the inverted repeats containing the original-specified π protein product of the original kanamycin resistance gene of the mini-Tn10 is replaced by a fragment containing a *SfiI*-P_{tr} cassette, appropriately modified by the addition of the *NotI* site and *MluI* adapters, which produced the pLODP_{tr}. This construction has unique *SfiI*, *NotI*, and *XbaI* cloning sites between the mini-Tn10 inverted repeats. The P_{tr} resistance marker (P_{tr}^r) of pLOFP_{tr} is exchanged by kanamycin
 15 resistance to produce plasmid pLOFKm.

In view of the foregoing, a need exists for the construction of stable recombinant *Z. mobilis* strains which are capable of fermenting xylose and arabinose, or both, to ethanol through the generation of stable genomic inserts which encode the enzymes which are necessary for xylose and arabinose catabolism. The strains should be free of antibiotic resistance and stable for more than 40
 20 generations in a non-selection media. It is also desirable for the strains to demonstrate a high specific rate of product formation at close to maximum theoretical product yield.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide transposable element and plasmid vectors
 25 useful for the stable integration of foreign structural genes, encoding enzymes selected from the group consisting of *xyIA*, *xyIB*, *tal/tkl*, and *araBAD* and at least one regulator gene for induction of the structural genes, into the *Z. mobilis* genome.

It is a further object of the invention to provide significantly different *Z. mobilis* strains capable of stable expression of the structural genes in a non-selection medium.

It is yet another object of the invention to provide significantly different *Z. mobilis* strains, having stable expression of the structural genes, but which are characterized by a high rate of specific product formation and conversion efficiency, when used as a bio-catalyst in a cellulose hydrolysate reaction mixture.

5 To overcome the problems associated with the related art and in accordance with the purpose of the invention, as embodied and broadly described herein, the present invention briefly includes a transposon for stable insertion of foreign genes into a bacterial genome, comprising: at least one operon having structural genes encoding enzymes selected from the group consisting of *xylA**xylB*, *araBAD* and *tal/iki*, and at least one promoter for expression of the structural genes in the bacterium,
 10 a pair of inverted insertion sequences, the operons contained inside the insertion sequences, and a transposase gene located outside of the insertion sequences. A plasmid shuttle vector for transformation of foreign genes into a bacterial genome, comprising: at least one operon having structural genes encoding enzymes selected from the group consisting of *xylA**xylB*, *araBAD* and *tal/iki*, at least one promoter for expression of the structural genes in the bacterium, and at least two
 15 DNA fragments having homology with a gene in the bacterial genome to be transformed..

The transposon and shuttle vectors are useful in constructing significantly different *Zymomonas mobilis* strains, according to the present invention, which are useful in the conversion of the cellulose derived pentose sugars into fuels and chemicals, using traditional fermentation technology, because they are stable for expression in a non-selection medium.

20 Additional advantages of the present invention will be set forth in part in the description that follows and in part will be obvious for that description or can be learned from practice of the invention. The advantages of the invention can be realized and obtained by the method particularly pointed out in the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

25 The accompanying drawing, which are incorporated in and which constitute a part of the specification, illustrate at least one embodiment of the invention and, together with the description, explain the principles of the invention.

Figure 1 is a plasmid map of Mini-Tn5 Tc in pGP704 containing the xylose assimilation operon according to the present invention.

Figure 2 is a series of plasmid maps illustrating the mini-Tn5 series constructs, according to the present invention.

Figure 3 is an illustration of the series of constructs resulting in the plasmid map of pUC*tal**tk**Sfi*.

5 Figure 4 is the results of the Southern analysis *tal/ikt-xyLAxylB* *Z. mobilis* transconjugates with *Tal* probe. C is the control plasmid mini Tn5 *tal/ikt-xyLAxylB*. λ H is the DNA size marker.

Figure 5 is the results of the Southern analysis of *tal/ikt-xyLAxylB* *Z. mobilis* transconjugates with *Tnp* probe. C is the control plasmid mini-Tn5 *tal/ikt-xyLAxylB*. λ H is the DNA size marker.

10 Figure 6 is a graph of the enzymatic activities for several isolates of the stable xylose fermenting *Z. mobilis* strain according to the present invention.

Figure 7 illustrates graphs of the fermentation profiles for four of the isolates (nos. 21, 22, 5, and 11) of Figure 6 and their performance in relation to the plasmid bearing strains 39673/pZB4 and BC1/pZB4L.

15 Figure 8 is a graphic representation which illustrates the stability of the stable xylose fermenting *Z. mobilis* strains C25 and D95 according to the present invention. The graph shows the stability of the plasmid-bearing and genomic integrated xylose-fermenting strains, using the ethanol process yield and xylose utilization parameters as indicators. Strains C25 and D95 of the present invention remained stable for more than 90 generations. The fermentation medium comprised RM with 1 glucose and 5% xylose and the temperature was constant at 30° C.

20 Figure 9 illustrates the biomass concentration (a), ethanol concentration (b), xylose utilization (c), and process yield compared to % xylose utilization results for batch fermentation of the genomic integrated xylose-fermenting strains, C25 and D95, in a RM medium containing 4.5% glucose, and % xylose at pH 5.0 and pH 5.5 at 30° C, according to the present invention.

25 Figure 10 is a map of the integrative plasmid pZB 1862-*ldhL*-*ara*. The *araBAD* is inserted in the *Not*I site of *ldhL* disrupting *ldh*. The construction is based on the replicative plasmid pZB 1862 of *Z. mobilis*.

Figure 11 is the plasmid map of Tn10G. *IS10_R* is the transposase gene. IR is the inverted repeat sequence of the transposon. *AraBAD* was inserted between two the two inverted repeats at the NotI sites.

Figure 12 shows the Southern analysis of the genomic integrated xylose/arabinose-fermenting *Z. mobilis* strains from homologous recombination using DIG-*ara* and DIG-*ldh* probes. AX1, 13, 23, 101, 104, and 107 are *araBAD* integrants. C25 is the host control pZB1862-*ldh*L-*ara* is the plasmid control isolated form DHS α . λ H is a molecular weight marker: 23, 9.4, 6.6 4.3, 2.3 and 2.0 kb. (Figure 12 includes Figs. 12A and 12B).

Figure 13 represents a Southern analysis of the genomic integrated xylose/arabinose-fermenting *Zymomonas* strains from transposon integration using DIG-*tnp* and DIG-*ara* probes. G5,6,11,15,17,14 and 19 are *araBAD* integrants. C25 us the host control. Tn10G is the plasmid control. λ H is a molecular weight marker: 23,9.4,6.6,4.3,2.3 and 2.0 kb. Figure 14 (a) is the *mp* *Pst*I digestion and Figure 14 (b) is the probe are *Pst*I digestion. (Fig. 13 includes Figs. 13A and 13B).

Figure 14 represents bar graphs of the enzymatic activities of the transketolase, transaldolase, xylose isomerase and xylulokinase of the genomic integrates strains. 206C/pZB301 is a plasmid control. 206C is a host control. C25 is the xylose-fermenting integrant. G8 is the xylose/arabinose fermenting integrant from Tn10 transposition. AX1 and AX101 are the xylose/arabinose fermenting integrants from homologous recombination.

Figure 15 represents bar graph results of the enzymatic activities of L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate-4 epimerase of genomic integrated strains. 206C/pZB301 is a plasmid control. 206C is a host control. C25 is the xylose-fermenting integrant. G8 is the xylose/arabinose-fermenting integrant form Tn10 transposition. AX1 and AX101 are the xylose/arabinose-fermenting integrants from homologous recombination.

Figure 16 represents a bar graph result of the ethanol process yields of the genomic integrated xylose and arabinose-fermenting *Zymomonas* strains on RMGXA (1:2:2%) at T=30° C, without pH control. These strains were inoculated form cultures at various generations on non-selective media.

Figure 17 represents a bar graph results of the xylose and arabinose utilization of the genomic integrated xylose and arabinose-fermenting *Zymomonas* strains on RM containing 1% glucose, 2

xylose and 2 arabinose at 30° C with pH control. These strains were inoculated from cultures at various generations on non-selective media.

Figure 18 is a line graphic representation of the fermentation performance of the genomic integrated xylose and arabinose-fermenting *Zymomonas* strains in RM containing 4% glucose, 4% xylose and 2% arabinose at pH 5.5 and 30° C.

DETAILED DESCRIPTION OF THE DRAWINGS

Unless specifically defined otherwise, all technical or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All US Patents are incorporated by reference as though fully set forth herein.

Reference now will be made in detail to the presently preferred embodiments of the invention, examples of which are illustrated in the accompanying drawings. For the examples described below, "plasmid-bearing strains" refers or relates to those strains and vectors described in the US Patents identified in the Description of the Related Art. "*Z. mobilis* genome, or genomic" means the genes which, *in toto*, specify all the expressed and potentially expressible with a given *Z. mobilis*.

EXAMPLES

Strains, Plasmids, and Media.

E. coli bacterial strains CC118λ(*pir*), CC118λ(*pir*) (mini-tn5Tc), SM10λ(*pir*), and plasmids pUC19, pLOF/Km, pUC18sfh, pUT/Tc containing minitransposon Tn5, Tn 10 and pUC18Not were obtained from Dr. K. Timmis, GBF - Gesellschaft Fur Biotechnologische Forschung mbH, Mascheroder weg 1 D - 38124 Braunschweig, Federal Republic of Germany. *E. coli* DH5α was used as a host for the construction of the plasmids. *E. coli* SM10λ*pir* was used as donor strain in mating experiments. Strains of *Z. mobilis* ATCC 39676 and its derivative, 206C (US Pat. No. 5,843,760) were used as recipients in accordance with the invention. Tn10-based plasmids were constructed and maintained in *E. coli* CC118.

E. coli strains were cultured in LB medium at 37° C. *Z. mobilis* strains were maintained anaerobically in RM (10 g/L yeast extract, 2 g/L KH₂PO₄) supplemented with 20g/L glucose, D-

xylose or L-arabinose, unless otherwise specified. All strains containing plasmids were grown in the presence of tetracycline (Tc) ((10 µg/ml in liquid *Z. mobilis*, and *E. coli*; 20 µg/ml in agar for *Z. mobilis* and 15 µg/ml in agar, or ampicillin (Ap), 100 µg/ml for *E. coli*). For regeneration and selection of *Z. mobilis* transformants or transconjugates, mating plates ((10 g/L yeast extract, 5 g/L tryptone, 2.5 g/L (NH₄)₂SO₄, 0.2 g/L K₂HPO₄ and 50 g/L sugar)) supplemented with tetracycline or nalidixic acid (20 µg/ml) were used. All agar plates were made with 15 g/L agar.

Recombinant DNA techniques.

Plasmid DNA isolation, restriction endonuclease digestion, ligation and transformation, agarose electrophoresis and other recombinant DNA techniques were carried out in accordance with published protocols, Sambrook *et al.*, (1989) *Molecular cloning: a laboratory manual*, Cold Spring Harbor laboratory press, Cold Spring Harbor, NY, or the respective reagent manufacture's instructions, were specified, and are well known in the art. Genomic DNA of *Z. mobilis* was extracted using three-milliliters of overnight cells resuspended in 250 ml of 50 mM Tris-50 mM EDTA buffer. The cells were treated with lysozyme at 37° C for 30 min 100 ml of 5% SDS solution and RNAase (final concentration equal to 20 ng/ml) were then added and incubated for an additional 30 min. A phenol/chloroform extraction was performed twice, to remove the proteins. Genomic DNA was recovered by ethanol precipitation.

Conjugation and transformation.

Plasmids were transferred from the donor strains *E. coli* SM10λpir or S17-1 into *Z. mobilis* strains by conjugation with a filter mating technique (Conway *et al.*, 1987). Plasmid DNAs were transformed into either *Z. mobilis* or *E. coli* cells by electroporation (Zhang *et al.*, 1995)).

Southern Blot analysis.

DNA was transferred onto a nylon membrane using a Stratagene Posi Blot pressure blotter. DNA probes Tc, xylB, Tnp, and Tal were digoxigenin-UTP labeled by Polymerase Chain Reactions (PCR). The following primers were used for DNA labeling:

Tc: 5'-TTGCTAACGCAGTCAGGC-3'

30 5'-GAATCCGTTAGCGAGGTG-3'

xylB 5'-TATGGGTTCAGCGGCATGAG-3'

5'-ATGGGCATGAGATCCATAGCC-3'

Tnp 5'-TCCTAACATGGTAACGTTC-3'

5'-CCAACCTTACCAGAGGGC-3'

Tal: 5'-CGTCTAAAAGATTTTAAGAAAGGTTTCGATATGACGGACAA
ATTGACC-3'

5'-CATTTTGACTCCAGATCTAGATTACAGCAGATCGCCGATC
ATTTTTTCC-3'

Prehybridization and hybridization were performed according to established protocols described in the Boehringer Mannheim hybridization kit.

EXAMPLE I

The following example illustrates the construction of Mini-Tn5Tc containing genes encoding the xylose assimilation enzymes and the conjugal transfer of this construct into *Z. mobilis* 206C and ATCC 39676. Mini-Tn5Tc containing genes encoding the xylose assimilation enzymes was constructed by inserting a *Pgap-xyLAxyLB* operon into the unique *NotI* site of Mini-Tn5Tc contained in plasmid pGP704. See, Figure 1. The *Pgap-xyLAxyLB* operons, were taken from plasmid pZB4 or pZB5, US Pat. Nos. 5,712,133 and 5,726,053. The resulting plasmids, designated Mini-Tn5Tc *xyLA/xyLB*(X4) and Mini-Tn5Tc *xyLAxyLB* (X5), were transformed into the donor strain *E. coli* SM10λ*pir* by electroporation and mated with either *Z. mobilis* ATCC 39676 and 206C strains. *Z. mobilis* 206C is disclosed in US Pat. No. 5,843,760 which is incorporated herein by reference. Nine *Zymomonas* Tc^r transconjugates were obtained from both SM10λ*pir* donors containing Mini-Tn5Tc*xyLAxyLB* (X4) and Mini-Tn5 Tc*xyLAxyLB* (X5) by selection on media containing Tc and nalidixic acid. See, Figure 2(b).

Genomic and plasmid DNA from the nine *Z. mobilis* Tc^r transconjugates was then subjected to Southern blot analyses. Genomic and plasmid DNA from the transconjugates was digested with SphI, which cuts in the hybrid transposon and in the xylose operon, and the blots were then hybridized to either a Tc probe, a XylB probe, or a transposase Tnp probe. From the autoradiograph it was determined that (1) the xylose operon *Pgap-xyLAxyLB* had been inserted into the *Zymomonas* genome, along with Tc^r gene; (2) only a single insertion had occurred for each of the transconjugates; (3) the insertions were each at a distinct location in the genome; (4) the Tc^r gene and XylB were not present the plasmid fraction; and (5) the *mp* transposase gene was not present in transconjugates.

Enzymatic analysis of the *Zymomonas* Tc^r *Pgap-xylA**xylB* Transconjugates was made in order to confirm the expression of xylose isomerase (XI) and xylulokinase (XK) in the *Zymomonas* Tc^r *Pgap-xylA**xylB* transconjugates. This analysis revealed that the enzymatic levels of xylose isomerase for the all the transconjugates was about one-half that of their plasmid counterparts. Similarly, about one-half of the xylulokinase activities of the plasmid-bearing strains was observed in the integrants. Both XI and XK activities, expressed from a single copy of the genes, in the *Zymomonas* genome, were considerably higher than was expected, when compared to that of the 10 copies per cell found in a plasmid-bearing strains.

EXAMPLE II (C25)

This example shows that to enhance the genetic stability of the recombinant *Z. Mobilis*, in the absence of antibiotic selection, the xylose assimilation genes *xylA* and *xylB*, encoding xylose isomerase and xylulokinase, and pentose phosphate pathway genes, *talB* and *tklA*, encoding transaldolase and transketolase, were introduced into the *Z. mobilis* genome, using mini-Tn5. Two operons containing *Pgap-xylA/xylB* and *Peno-talB/tklA* were assembled in mini-Tn5 and the resulting plasmid was conjugated into *Z. mobilis*. With the help of the transposase located outside of the mini-Tn5 cassette, single copies of the *Pgap-xylA**xylB* and *Peno-talB/tklA* were inserted into the *Z. mobilis* genome, as shown by Southern hybridization. Enzymatic analysis of xylose isomerase, xylulokinase, transaldolase and transaldolase indicated that all the genes were coordinately expressed and that the integrated strains produced about 30-70% of the enzymatic activities of the plasmid-bearing strains. These enzymatic levels were sufficient for the organism to grow and ferment xylose to ethanol.

To facilitate the cloning process, the *Bgl*II fragment containing the operon *Peno-talB/tklA* was inserted into the *Bam*HI site of a newly constructed auxiliary plasmid, pUSCfiMCS as shown in Figure 3. The auxiliary plasmid, pUSCfiMCS, was constructed by inserting a *Eco*R I-*Hind* III fragment containing the multicloning sites from pUC19 into pUCSfi. *PUCtal/tkt* was then constructed from pUCpfiMCS and pUCtal/tktSfi, as shown in the Figure.

Referring now to Figure 2, the *Peno-talB/tklA* was then excised, from pUCtal/tktSfi, as a *Sfi*I fragment and was used to clone into mini-tn5-Tc-*xylA**xylB*. As shown in the Figure, the Tc^r gene is flanked by *Sfi*I sites on the Tn5-Tc-*xylA**xylB* cassette. Mini-tn5-Tc-*xylA**xylB* was partially and completely digested with *Sfi* I and ligated to the *Peno-talB/tklA**Sfi* fragment, as shown in Figure 2(c). The partial digestion yielded a plasmid containing the Tc^r gene, designated as mini-tn5- Tc *tal/tkt*-

*xylA**xylB* ((Fig. 2(c)), while complete digestion yielded a plasmid, according to the invention herein, without the *Tc^r* gene, designated as miniTn5-*tal/kt-xylA**xylB*. See Figure 2(d).

Both plasmids were transformed into the donor strain *E. coli*, S17-1 and mated with *Z. mobilis* 206C. The resulting transconjugates were selected on mating media containing glucose, *Tc*, and nalidixic acid for miniTn5-*tal/kt-xylA**xylB*-*Tc*. For mini-tn5-*tal/kt-xylA**xylB*, the transconjugates were directly selected on mating media, containing xylose and nalidixic acid. A number of *Tc^r* transconjugates (glucose-grown) were obtained for mini-tn5 *tal/kt-xylA**xylB*-*Tc*. Several xylose-grown transconjugates were obtained for mini-tn5-*tal/kt-xylA**xylB*.

Preliminary batch cultures were tested statically, at 30° C without pH control in bottles with 80 ml RM containing 2% xylose for their fermentation dynamics. Colonies, taken from RM+2% xylose plates and were cultured in an RM 2% xylose medium overnight at 20° C until the culture had reached the stationary growth phase (optical density₆₀₀ = 0.1 at 500 nm), were used as the inoculum. Xylose and ethanol were analyzed using a Hewlett-Packard 1090L HPLC equipped with an HP 1047 A refractive index detector and a Biorad HPX-87H organic acid analysis column operating at 65° C, with a 0.01 N sulfuric acid mobile phase flow rate of 0.6 ml/min. The ethanol yield was calculated using either the weight of sugar fermented or the total available sugar in the medium. The maximum theoretical yield was based on 0.51 g ethanol/ g xylose.

Southern bolt analysis of genomic and plasmid DNA samples from the *Z. mobilis* transconjugates was made in order to investigate whether transposition of the mini-tn5 *tal/kt-xylA**xylB* cassette had occurred. Genomic and plasmid DNA's prepared from four transconjugates of mini-tn5 *tal/kt-xylA**xylB* were digested with *Sph*I, which cuts twice within the cassette yielding one fragment with *Tal* probe homology. The blots were then hybridized to either *Tal* probe or *Tnp* probe. The autoradiograph in Fig. 4 shows that one unique band greater than 4kb (the size of *Peno-talB/ktA*), which is adjacent to *Z. mobilis* DNA, was detected from all the genomic DNA preparations when hybridized with *Tal* probe. Three of the samples (nos. 22,23, and 24 possibly siblings) also showed a band in the plasmid fraction, suggesting that the integration had occurred in the native plasmid. Clearly integration had occurred in the *Z. mobilis* genome for sample no. 21, and only one copy was inserted. Hybridization of genomic and plasmid DNA samples from these transconjugates with a *Tnp* probe (Fig. 5) revealed a lack of homology between the sample DNA's and *Tnp* probe. These results suggested that the xylose assimilation and pentose phosphate pathway genes, along with *Tc^r* gene, had been transposed into the *Z. mobilis* genome, and that only a singly

insertion occurred in each of the transconjugates, and the insertions were at distinct locations in the genome.

Xylose isomerase, xylulokinase, transaldolase and transketolase activities were measured as previously described in (Zhang *et al.*, 1995), for the *Z. mobilis* transconjugates. As set forth above, it had been observed that expression of a single *Pgap-xylA_{xy}lB* copy on the genome was about one-half that of the XI and XK enzymatic yielded for the plasmids-bearing strains. However, when enzymatic analysis was done to confirm whether transaldolase (TAL) and transketolase (TKT) had been expressed in the *Zymomonas tal/tki-xylA_{xy}lB*-Tc and *tal/tki-xylA_{xy}lB* transconjugates, as shown in Figure 6, it was revealed that the enzymatic levels of TAL, for the all the transconjugates (nos. 4,5,17,18,21,23, and 24), was about 50-70% that of their plasmid counterparts. About 30-70% of the TKT activities of the plasmid-bearing strains was observed in the integrants. While the plasmid integrants had slightly elevated TAL and TKT activities, both TAL and TKT activities, expressed from a single copy of the genes in the *Zymomonas* genome, were considerably higher than was expected when compared to that of the 10 copies per cell found on a plasmid-bearing strain.

All four of the *tal/tki-xylA_{xy}lB* *Zymomonas* integrants (nos. 21, 22, 5, and 11) were able to grow on xylose. Preliminary fermentation studies for these integrants was then made using a 2% xylose substrate, at 30° C. The fermentation profiles, for these integrants, is shown in Figure 7. In the Figure, the growth and sugar utilization rates of *Z. mobilis* integrant no. 21 was slower than that of the plasmid bearing strain 39676/pZB4. However, most of the *Z. mobilis* integrants (nos. 22,5, and 11) were comparable to the plasmid-bearing strains. All the integrants produced ethanol from xylose, at 92% of the maximum theoretical product yield.

Stability of these genomic integrated strains and three plasmid bearing strains was measured in a non-selective medium. All strains were cultured in an RMG medium, and serially transferred about every 10 generations daily. At every 40 generations, the cells were used to inoculate a flask of RM media containing 1% glucose, and 5% xylose in order to measure their ability to ferment xylose to ethanol. Ethanol process yields and xylose utilization rates were used as the milestones for stability. Two of the genomic integrated strains demonstrated stability for more than 90 (C25 and D95) generations, while the plasmid-bearing strains (206/pZB4, 206/pZB4L and BC1pZB4L) were stable for to about 40 generations. See, Figure 8.

The fermentation performance of strains C25 and D95 analyzed in an RM medium containing a different concentrations of total glucose and xylose (1% glucose and 5% xylose; 3% glucose and

3% xylose; and 4.5% glucose and 4.5% xylose) under pH controlled conditions. As shown in the Figure, strain C25 demonstrated much better xylose utilization and ethanol process yields at both pH 5, and pH 5.5 in RM containing 4.5% glucose and 4.5% xylose better than D95. Consequently, in the subsequent examples the three arabinose assimilating genes (*araBAD*) were integrated into the C25 genome.

EXAMPLE III (AX)

The following example demonstrates the introduction of the arabinose assimilation enzymes in the genome of C25 through homologous recombination via *ldh* and transposition using mini-transposon Tn10. Plasmid pZB1862-*ldhL*-*ara*, described below, was used to transform *Z. mobilis* or *E. coli* by electroporation. Transformants were selected on mating plates supplemented with glucose and tetracycline. Tc^r colonies were further confirmed to be Ara⁺Xyl⁺ by growth on RM supplemented with xylose or arabinose (RMS and RMA). Plasmid Tn10G, also described below, was transferred from an *E. coli* SM10λ*pir* donor to *Z. mobilis* C25 by conjugation with a filter mating technique (Conway *et al.*, 1987). The resulting transconjugates were selected on mating plates containing arabinose.

A. Construction of pZB1862-*ldhL*-*ara* and integration in C25 using homologous recombination.

Previous attempts to integrate *araBAD* in the *Zymomonas* genome, using a 1-kb *ldh* fragment as the homologous region, did not succeed. In order to increase the recombination frequency, a larger homology region was used. A 2.5-kb DNA fragment, which includes *ldh* and the flanking region was amplified using *Pfu* PCR. The primers were designed based on the DNA sequence of *Z. mobilis* CP4, published in Yamano I., (1993) *Journal of Bacteriology*, Vol. 175, 3926-3933.

Although a 2.5-kb fragment was expected from PCR, according to the published sequence, a 3.4-kb fragment was obtained instead. After digesting the 3.4-kb fragment with *Bam*HI, two fragments (2.5 and 0.9 kb) were obtained. Both fragments were tested by PCR, using primers designed to anneal to only the *ldh*. The 2.5-kb fragment produced a PCR product of the correct size, whereas the 0.9-kb fragment did not, indicating that the former contained the *ldh* sequence. Therefore, the 2.5-kb *Bam*HI fragment (designated *ldhL*) was cloned and used as the homologous region for gene

integration into C25.

The *ldhL* fragment and digoxigenin (DIG)-labeled *ldh*, *ara*, and *tnp* probes were amplified by PCR using either Pfu (Stratagene, La Jolla, CA) or Taq DNA polymerase (Qiagen, Valencia, CA). DIG-UTP was purchased from Boehringer Mannheim, Indianapolis, IN. A I probe was used to probe for IS10_R, the transposase gene of Tn10. PCR products for *ldhL*, *ldh*, *ara*, and *tnp* are 2.5, 1, 1.4 and 0.8 kb, respectively. The following primer sequences were used:

ldhL: 5'-TCGCGGATCCTCTATCCCTTTATTTTCTATCCCCATCACCTCGG-3'

5'-TCGCGGATCCGCGGGCTGACATACATCTTGCGAATATAGGG-3'

DIG-*ldh*: 5'-TCGCGGATCCGTCTATGCGCGTCGCAATATTCAGTTCC-3'

5'-TCGCGGATCCGTCTGCTTGTCTATTAAACAAGCGCATCCGGC-3'

DIG-*ara*: 5'-CTAACATGTTGACTCCTTCTCTAGACTTAGCG-3'

5'-GTTGAAACCGCTGGGCACCACGC-3'

DIG-*tnp*: 5'-CGCACTACACGGTCGTTCTGTTAC-3'

5'-GGTTGCAGCCACGAGTAAGTCTTC-3'

For cloning purposes, a *Not*I site was introduced in *ldhL* by insertion of an oligonucleotide 5'-CATGCGCGGCCGCC-3' at *Nco*I site, which is located in the middle of the *ldh* gene. The new *Not*I site was approximately 1.4 and 1.1 kb from either end of *ldhL*. A *Bam*HI fragment of *ldhL* (2.5 kb) containing the *Not*I site was ligated into pZB1862 at a *Bc*II site. Finally, a 4.4-kb *Pgap-araBAD*, isolated from pZB206 (US Pat. Nos. 5,712,133 and 5,726,053), was cloned into the *Not*I site, of *ldhL*, to form the integrative plasmid, pZB1862-*ldhL*-*ara*. See, Figure 10.

The *Pgap-araBAD* operon, containing the three arabinose-assimilating genes, was integrated into the *ldh* site in the C25 genome through homologous recombination. To integrate the *araBAD* genes into the genome of C25, pZB1862-*ldhL*-*ara* was constructed in *E. coli* DH5 α . The plasmid pZB1862-*ldhL*-*ara* was transferred into C25 by electroporation. The Tc resistant transformants were selected and tested for growth on arabinose. During propagation of the transformants, *Pgap-araBAD* could be integrated in the genome of C25 by the replacement of *ldhL* with the *ldhL*'-*araBAD*-*ldhL*' cassette (from the plasmid) through homologous recombination.

To enrich and isolate the integrants, plasmid curing was conducted for the transformants. Plasmid pZB1862-*ldhL*-*ara* will replicate in *Z. mobilis*. However, *Z. mobilis* tends to lose foreign plasmids at sub-optimal growth conditions (e.g. 37° C). Using this characteristic, curing of pZB1862-*ldhL*-*ara* was achieved by subculturing C25 transformants at 37° C in the absence of Tc for several transfers. Cultures from each transfer were constantly monitored for the loss of the

plasmid. By the third transfer, 100% of the cells became Tc^r, indicating a loss of the plasmid. Cultures from the 3rd, 4th, 5th, and 6th transfers were inoculated in RM containing arabinose (RMA), at 30° C, to enrich the growth of potential *Pgap-araBAD* integrants. The enriched cells were transferred to RMG plates and replica-picked onto RMA, RMX, and RMGTc plates. Several integrants (AX) with the phenotype of Xyl⁺Ara⁺Tc^r were subjected to further analysis, as described below. These integrants were able to use either xylose or arabinose as a sole carbon source.

B. Construction of Tn/0G and Conjugation into C25.

Mini-Tn5 was used for constructing C25 with *Peno-ial/ikt* and *Pgap-xyLAB* operons. Although the transposase gene did not exist in C25, mini-Tn/0 was used for the subsequent integration of *Pgap-araBAD* to avoid any possible incompatibility between the same transposons. Plasmid Tn/0G (Fig. 11) was constructed based on the Tn/0-based delivery plasmid, pLOFKm. The Km^r gene was replaced by a *NotI* fragment of *Pgap-araBAD*, isolated from pZB206. Tn/0G was constructed and maintained in *E. coli* C118. The plasmid was then transferred into the mating donor, *E. coli* SM10λpir for conjugation with *Z. mobilis*. Since Tn/0G is a suicide plasmid in *Z. mobilis*, only transconjugates with *araBAD* integration were able to grow on mating plates, supplemented with arabinose. The *E. coli* SM10λpir donor was inhibited, in the plates, by the presence of nalidixic acid. Transconjugates appeared on mating/ara plates in 7 days. Colonies were replica-picked onto RMA and RMX to confirm their phenotypes. Eighty-six percent of the colonies picked were Xyl⁺Ara⁺. Twenty colonies from the pick-plates were cross-transferred to different plates (from xylose plates to arabinose plates or vice versa). Sixty percent of those colonies remained Xyl⁺Ara⁺. Twenty colonies were analyzed in a preliminary Southern hybridization (data not shown). Using the *mp* probe, about 50% of the strains contained the transposase gene in the genome. Eight integrants were then subjected to detailed analysis by Southern hybridization.

Integration of *Pgap-araBAD* in *ldh* of C25 was confirmed by Southern hybridization, for the pZB1862-*ldhL-ara* integrants DNA using the DIG-labeled *ara* and *ldh* probes. See, Figure 12(a) and (b). There is only one *PstI* site on pZB1862-*ldhL-ara* and it is located in *Pgap-araBAD*. Therefore, one hybridization band (12.9 kb) from the *PstI*-digested plasmid was expected, using the *ara* probe. With *Pgap-araBAD* integrated in the genome two bands generated by the *PstI* site in *Pgap-araBAD* and the adjunct *PstI* sites on the genome located outside the *Pgap-araBAD* were expected. The results from Figure 13(a) clearly showed that two bands from the total DNA preparation hybridized

with the *ara* probe and demonstrated integration of *Pgap-araBAD*. The lack of hybridization bands from plasmid DNA of integrants indicated that integration had occurred on the genome, rather than on native plasmids. To show that the *ldh* was disrupted by the *Pgap-araBAD* integration, the same DNA was transferred and hybridized with the *ldh* probe. As expected, the hybridization patterns for the integrants were exactly the same on both blots, except for C25, as shown in Figure 12 (b). The total DNA from the host strain, C25, used for *Pgap-araBAD* integration, which has an intact *ldh*, showed only one band. The results confirmed that *araBAD* was integrated in *ldh* of C25.

Figures 13(a) and (b) show the Southern hybridization results using DIG-labeled *Tnp* and *ara* probes, respectively, for the eight *Pgap-araBAD* transposon integrants generated by Tn10 transposition. DNA was digested with a PstI restriction enzyme. PstI cuts Tn10G into two fragments (7.7 and 5.9 kb) and only the 5.9-kb fragment carries the transposase gene (*IS10_R*). Both fragments hybridized with an *ara* probe. According to the blots, only G8, G11, G15 and GH17 were *ara*-positive and *tnp*-negative. Different band patterns indicated that *Pgap-araBAD* was integrated at different loci in the genome. Although the transposase gene was not expected to remain in the genome of the integrants, four strains (G5, G6, G14 and G19), out of the eight, contained the transposase gene. Furthermore, G14, and G19 contained the transposase gene on the plasmid. With an *ara* probe, two bands from the integrants were expected. However, only single bands were observed. To solve this ambiguity, PCR was conducted, for the integrants, using *ara* primers and it was confirmed that all eight integrants contained *Pgap-araBAD* in the genome (data not shown).

Xylose isomerase (XI), xylulokinase (XK), L-arabinose isomerase (L-AI), L-ribulokinase (L-RK), L-ribulose-5-P-4-epimerase (L-Repi), transketolase (TKT) and transaldolase (TAL) were assayed, using cell-free extracts of the *Z. mobilis* integrants and control strains, according to Zhang, *et al.*, 1995; and Deanda *et al.*, 1996, with minor modifications. Cell-free extracts were prepared by collecting the cultures at late-log phase (30° C, OD₆₀₀ approximately 1.2), washing once with sonication buffer (10 mM Tris-HCl, pH 7.6 . 10 mM MgCl₂) and sonicating. The cell debris was removed by centrifugation (14,000 rpm, 45 min 4° C). In the L-AI assay, the volumes of timed samples were scaled down by half (50µl), 70% H₂SO₄ (1.5 ml) and 0.12% carbazole (50µl). All of the tubes were maintained in a 25° C water bath, both before and after the addition of 70% H₂SO₄, until reading the absorbency. The samples were taken at 0,5,10, and 20 min during the reaction.

Although the integrants, from both homologous recombination and transposon integration, were able to grow on D-xylose and L-arabinose, the expression level of the integrated genes it was determined by measuring enzymatic activity. Isolates C25/AX1, C25/AX101, and C25/G8 were chosen for the enzymatic assays because they were the most stable integrants, as determined in the stability studies described below. The results of the enzymatic assays are summarized in Figures 14 and 15. For all assays (with and exception of xylulokinase), integrants showed positive activities as compared to the controls (C25 and/or 206C). It is believed, at this moment, that the low activity of XK for the integrants, might be due to experimental error, the nature of the assay, or both. In most assays, (excluding L-ribulokinase and xylose isomerase), the integrants showed lower activities than the plasmid-bearing strain (206C/pZB301). This is presumable related to the copy number of the genes.

For stability studies, the cultures were inoculated into test tubes containing RMG, incubated overnight at 30° C, and transferred daily to RMG tubes. The inoculum was controlled to allow transfer every 10 generations. At every 40 generations, the cells were used to inoculate flasks, containing a mixture of sugars, to test the fermentation capabilities on the sugars without pH control at 30° C. Batch fermentation studies were performed at 30° C with pH control in Bio-StatQ chemostats, a trademark of B. Braun, Allentown, PA., using 500 ml as the working volume. The pH was automatically controlled with 2N KOH. Initial sugar concentration and pH varied between each batch, depending on the culture conditions. All the sugars used were reagent grade. Samples were taken periodically throughout the fermentation, and analyzed for sugars, ethanol and by-products with HPLC, as described previously (Zhang 1995). Optical density, at 600 nm (OD600), was measured in order to monitor cell growth. Ethanol yield was based on the amount of total available sugar.

Several genomic integrated xylose and arabinose fermenting *Z. mobilis* strains developed through both homologous recombination and transposition were studied for their stability in a non-selective medium (RMG). These strains were cultured in RMG medium and serially transferred, daily, after about 10 generations. After every 40 generations, the cells were used to inoculate a flask containing 1% glucose and 2% xylose and 2% arabinose for examination of their ability to ferment xylose and arabinose to ethanol. Ethanol process yields, and xylose and arabinose utilization rates, were used as the stability trait. Two of the isolates remained stable for 160 generations. See, Figures 16 and 17. Three integrated strains and a plasmid-bearing strain were further tested for fermentation performance, in a media containing a mixture of 4% glucose, 4% xylose, and 2% arabinose at pH 5.5

and 30° C. As shown in Figure 18, all three strains utilized glucose, xylose and arabinose in 72 hours, while the plasmid-bearing strains still had 6 g/L residual arabinose. However, the integrated strains produced more xylitol (4 g/l) than the plasmid bearing strain (1 g/L). The two homologous recombination AX1 and AX101 strains did not produce lactate because the lactate dehydrogenase gene was inactivated through the gene integration. The process yields (about 83% of theoretical) of the integrated strains were very similar to the plasmid bearing strain. Moreover, the integrated strains grew to a greater cell densities, which is probably due to the lesser metabolic burden associated with having only a single copy of the seven genes.

While the present invention has been described in connection with the illustrated embodiments, it will be appreciated and understood that modifications may be made without departing from the true spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: MIDWEST RESEARCH INSTITUTE
- (ii) TITLE OF INVENTION: STABLE ZYMOMONAS MOBILIS XYLOSE AND ARABINOSE FERMENTING STRAINS
- (iii) NUMBER OF SEQUENCES: 16
- (iv) CORRESPONDENCE ADDRESS:
FILE REFERENCE: NREL 00-14 CA
- (v) COMPUTER READABLE FORM:
(D) SOFTWARE: PatentIn Ver. 2.1
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: CA 2,304,929
(B) FILING DATE: 2000-05-02

(2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown
- (vi) ORIGINAL SOURCE:
(A) ORGANISM: Artificial Sequence
- (ix) FEATURE:
(D) OTHER INFORMATION: Description of Artificial Sequence:
Tc primer for DNA labeling
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ttgctaacgc agtcaggc

18

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown
- (vi) ORIGINAL SOURCE:
(A) ORGANISM: Artificial Sequence
- (ix) FEATURE:
(D) OTHER INFORMATION: Description of Artificial Sequence:
Tc primer for DNA labeling
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

gaatccgtta gcgaggtg

18

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Artificial Sequence
- (ix) FEATURE:
 - (D) OTHER INFORMATION: Description of Artificial Sequence:
xylB primer used for DNA labeling
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

tatgggttca gcggcatgag

20

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Artificial Sequence
- (ix) FEATURE:
 - (D) OTHER INFORMATION: Description of Artificial Sequence:
xylB primer for DNA labeling
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

atgggcatga gatccatagc c

21

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Artificial Sequence
- (ix) FEATURE:
 - (D) OTHER INFORMATION: Description of Artificial Sequence:
Tnp primer for DNA labeling
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

tcctaacaatg gtaacgttc

19

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid

- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Artificial Sequence

- (ix) FEATURE:
 - (D) OTHER INFORMATION: Description of Artificial Sequence:
Tnp primer for DNA labeling

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

ccaaccttac cagagggc

18

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 48
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Artificial Sequence

- (ix) FEATURE:
 - (D) OTHER INFORMATION: Description of Artificial Sequence:
Tal primer for DNA labeling

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

cgtctaaaag attttaagaa aggttttcgat atgacggaca aattgacc

48

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 49
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Artificial Sequence

- (ix) FEATURE:
 - (D) OTHER INFORMATION: Description of Artificial Sequence:
Tal primer for DNA labeling

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

cattttgact ccagatctag attacagcag atcgccgatc attttttcc

49

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 45
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown

- (vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE:

(D) OTHER INFORMATION: Description of Artificial Sequence:
Primer for amplification of ldhL gene fragment
using PCR

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

tcgcggatcc tctatccctt tatttttcta tccccatcac ctcg

45

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE:

(D) OTHER INFORMATION: Description of Artificial Sequence:
Primer for amplification of ldhL gene fragment
using PCR

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

tcgcggatcc gcggctgaca tacatcttgc gaatataggg

40

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE:

(D) OTHER INFORMATION: Description of Artificial Sequence:
Primer for amplification of DIG-ldh gene fragment
using PCR

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

tcgcggatcc gtctatgcgc gtcgcaatat tcagttcc

38

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

- (ix) FEATURE:
 - (D) OTHER INFORMATION: Description of Artificial Sequence:
Primer for amplification of DIG labeled ldhL gene
fragment using PCR

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

tcgcg gatcc gtcgcttgtc tattaaacaa gcgcattccgg c 41

- (2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Artificial Sequence

- (ix) FEATURE:
 - (D) OTHER INFORMATION: Description of Artificial Sequence:
Primer for amplification of DIG labeled ara gene
fragment using PCR

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ctaacatggt gactccttct ctagacttag cg 32

- (2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Artificial Sequence

- (ix) FEATURE:
 - (D) OTHER INFORMATION: Description of Artificial Sequence:
Primer for amplification of DIG labeled ara gene
fragment using PCR

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

gttgaaaccg ctgggcacca cgc 23

- (2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Artificial Sequence

- (ix) FEATURE:
(D) OTHER INFORMATION: Description of Artificial Sequence:
Primer for amplification of DIG labeled tnp gene
fragment using PCR

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

cgcactacac ggctcgttctg ttac

24

- (2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

- (vi) ORIGINAL SOURCE:
(A) ORGANISM: Artificial Sequence

- (ix) FEATURE:
(D) OTHER INFORMATION: Description of Artificial Sequence:
Primer for amplification of DIG labeled tnp gene
fragment using PCR

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ggttgcagcc acgagtaagt cttc

24

We claim:

1. A transposon for stable insertion of foreign genes into a bacterial genome, comprising:
at least one operon having structural genes encoding enzymes selected from the group consisting of
*xylA**xylB*, *araBAD* and *tal/tkt*, and at least one promoter for expression of the structural genes in the
5 bacterium, a pair of inverted insertion sequences, the operons contained inside the insertion
sequences, and a transposase gene located outside of the insertion sequences.
2. A plasmid vector containing the transposon of claim 1.
3. A bacterium transformed with the plasmid of claim 2.
4. The bacterium of claim 3 wherein the bacterium is *Z. mobilis*.
- 10 5. The bacterium of claim 3 wherein the transposon is a Tn5 or Tn10 derivative.
6. The bacterium of claim 4 wherein the operon comprises *xylA**xylB* and *tal/tkt*.
7. The bacterium of claim 4 wherein the operon comprises *araBAD* and *tal/tkt*.
8. The bacterium of claim 4 wherein the promoter is selected from the group consisting
of *P_{eno}* and *P_{gap}*.
- 15 9. A plasmid vector for transformation of foreign genes into a bacterial genome,
comprising: at least one operon having structural genes encoding enzymes selected from the group
consisting of *xylA**xylB*, *araBAD* and *tal/tkt*, at least one promoter for expression of the structural
genes in the bacterium, and at least two DNA fragments having homology with a DNA sequence in
the bacterial genome to be transformed.
- 20 10. A bacterium transformed with the vector of claim 9.
11. The bacterium of claim 6 transformed with the vector of claim 9 wherein the first
operon of the vector is consisting essentially of *araBAD*.
12. The bacterium of claim 7 transformed with the vector of claim 10 wherein the first
operon of the shuttle vector is consisting essentially of *xylA**xylB*.
- 25 13. The vector of claim 9 wherein the DNA fragments having homology are *ldhL* and the
DNA sequence in the bacterial genome to be transformed is *ldh*.
14. The bacterium of claim 10 wherein the bacterium is *Z. mobilis*.
15. The bacterium of claim 6 wherein expression of the structural gene product is stable
for at least 45 generations.

16. The bacterium of claim 13 wherein expression of the structural gene products is stable for at least 45 generations.

17. A vector pZB1862-ldhL-*ara* ATCC accession no. _____.

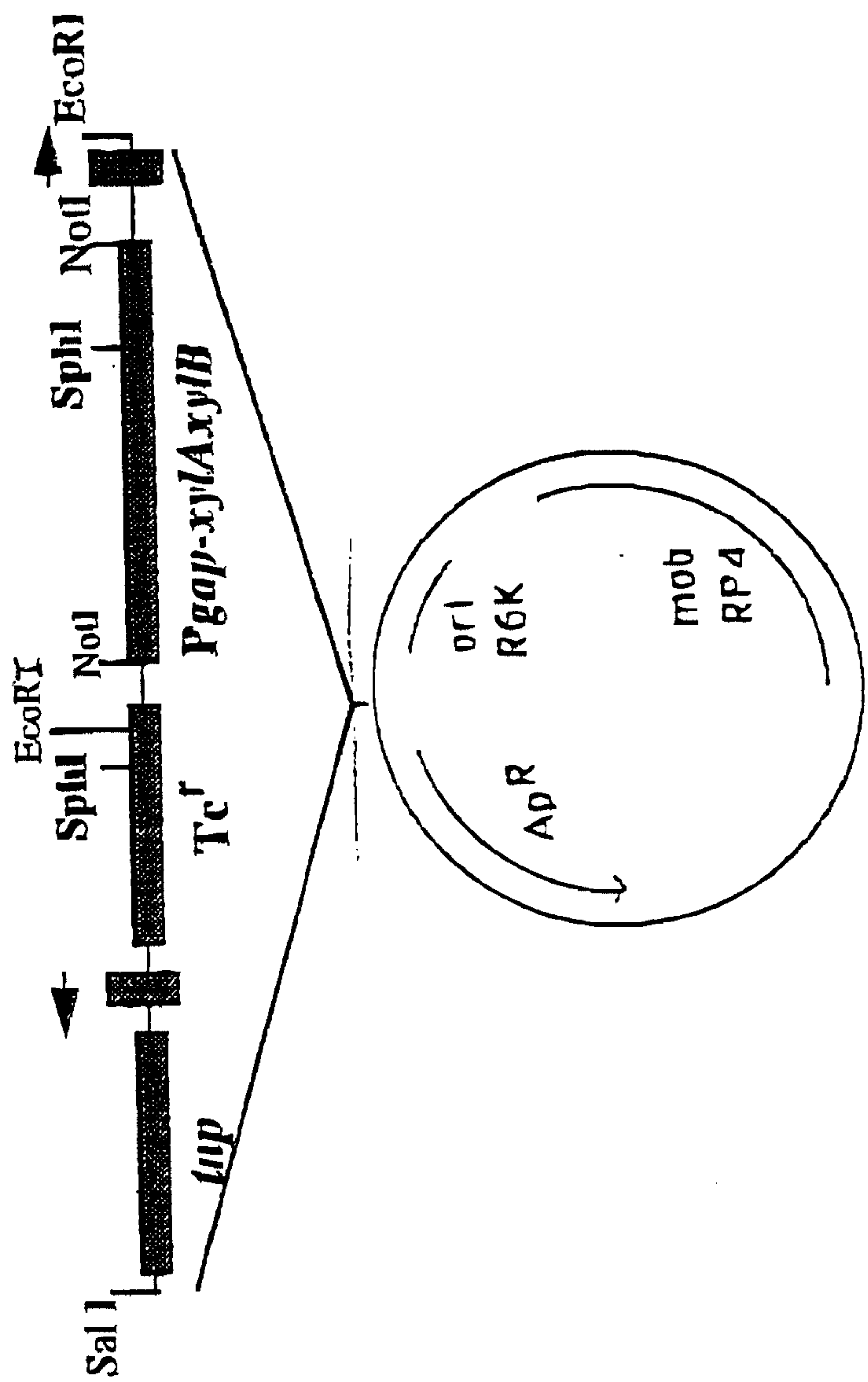
18. A transposable element mini-Tn5-*tal/kt-xylA**xylB*.

5 19. A bacteria strain G8 ATCC accession no. _____.

20. A bacteria strain C25 ATCC accession no. _____.

21. A bacteria strain AX ATCC accession no. _____.

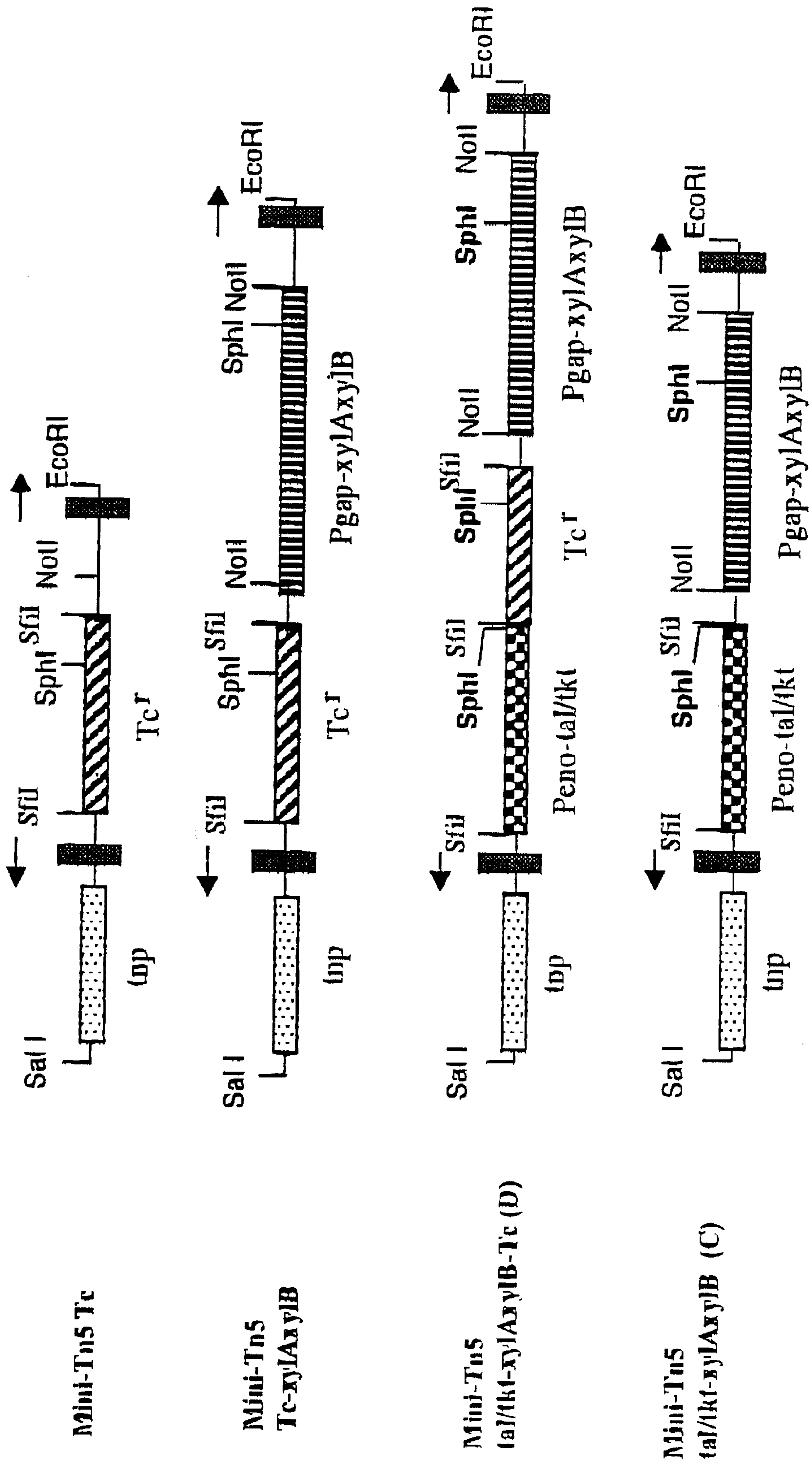
22. A reaction mixture comprising a fermentable medium, the medium including xylose or arabinose and a bacterium as in any one of the preceding bacterium claims.



Mini-Tn5 Tc with Xylose Assimilation Operon.

FIG 1

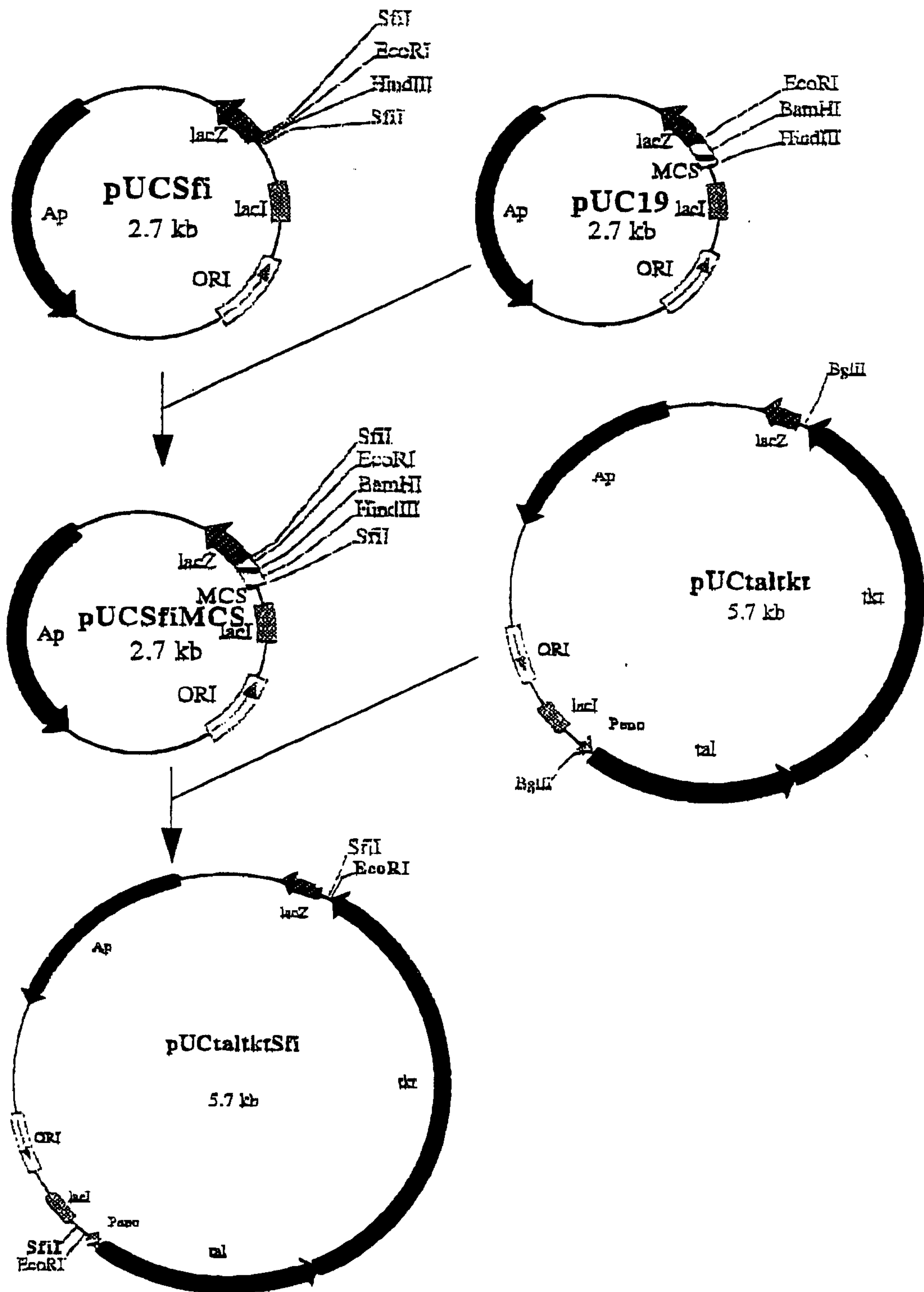
Feulayson & Seaglehurst
PATENT AGENTS



Plasmid maps for mini Tn5 series constructs.

FIG. 2

Fealayan & Senglehurst
PATENT AGENTS

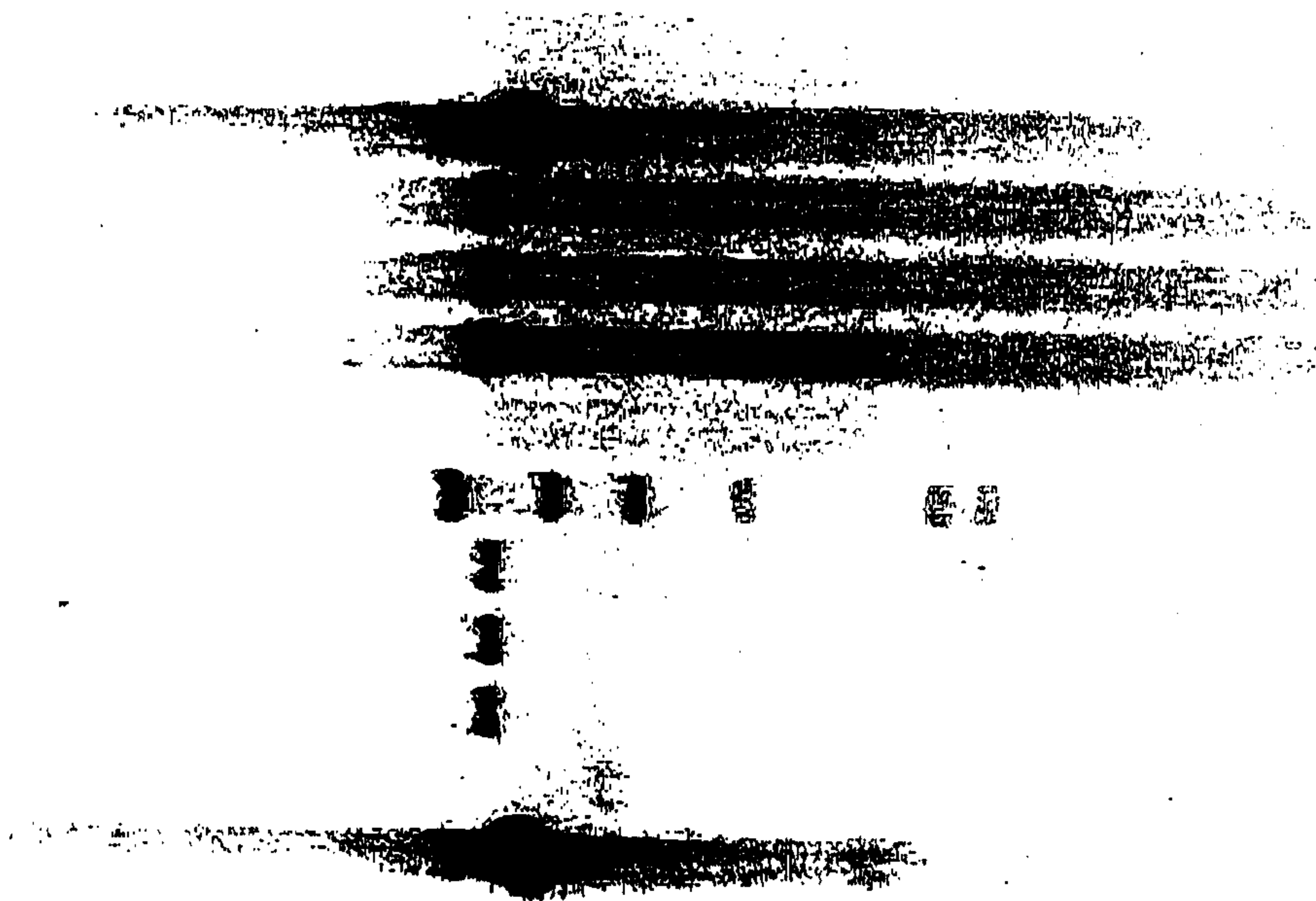


Construction of pUCtaltktSfi.

FIG. 3

Kenley & Penzance
PATENT AGENTS

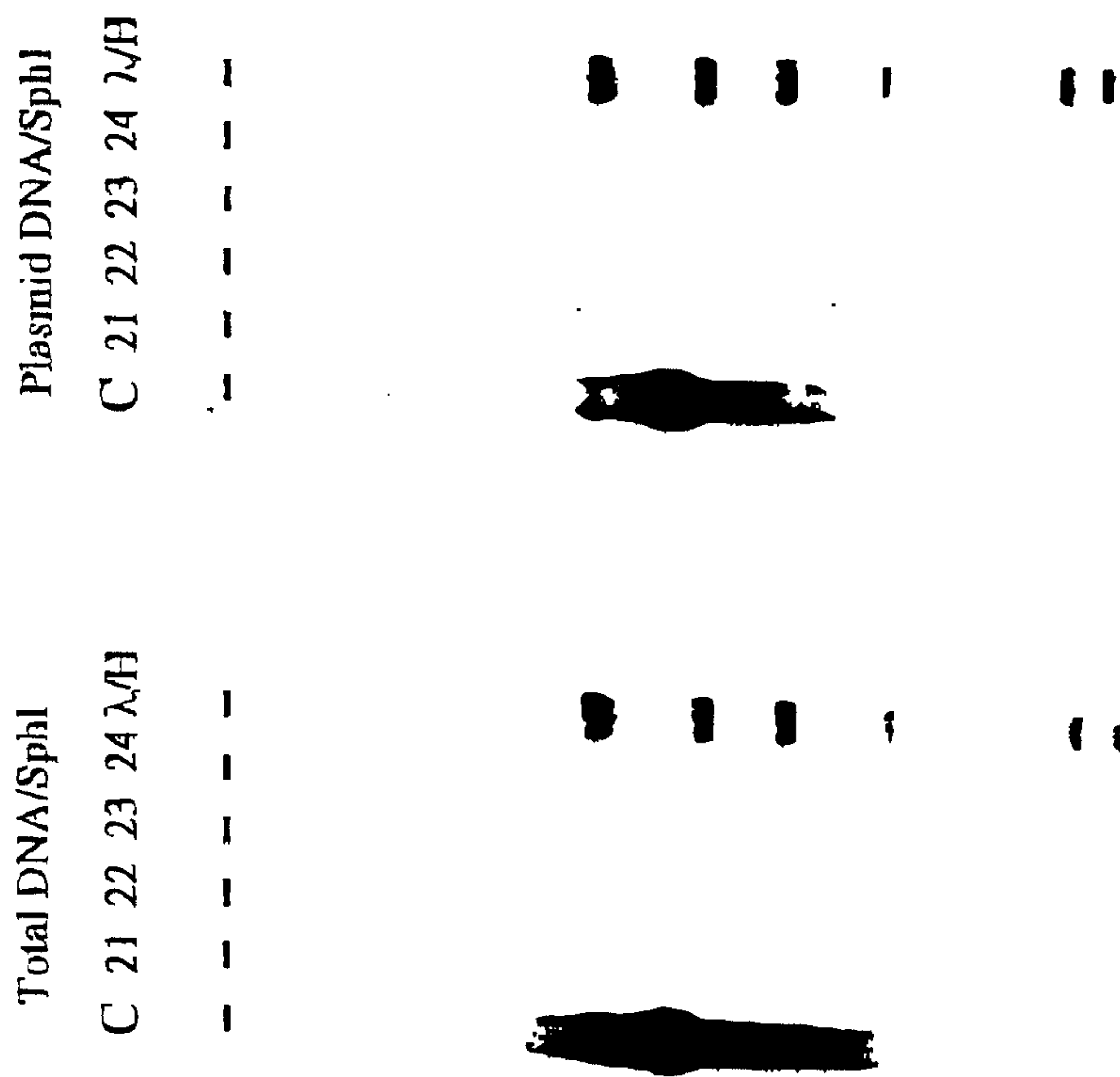
Total DNA/SphI		Plasmid DNA/SphI	
C	21	21	C
22	22	22	24
23	23	23	23
24	24	24	22
	λ/H		21



Southern analysis of tal/tkt-xy1Axy1B *Z. mobilis* transconjugates with Tal probe.
 C: control plasmid mini Tn 5 tal/tkt-xy1Axy1B. λ/H: DNA size marker.

FIG. 4

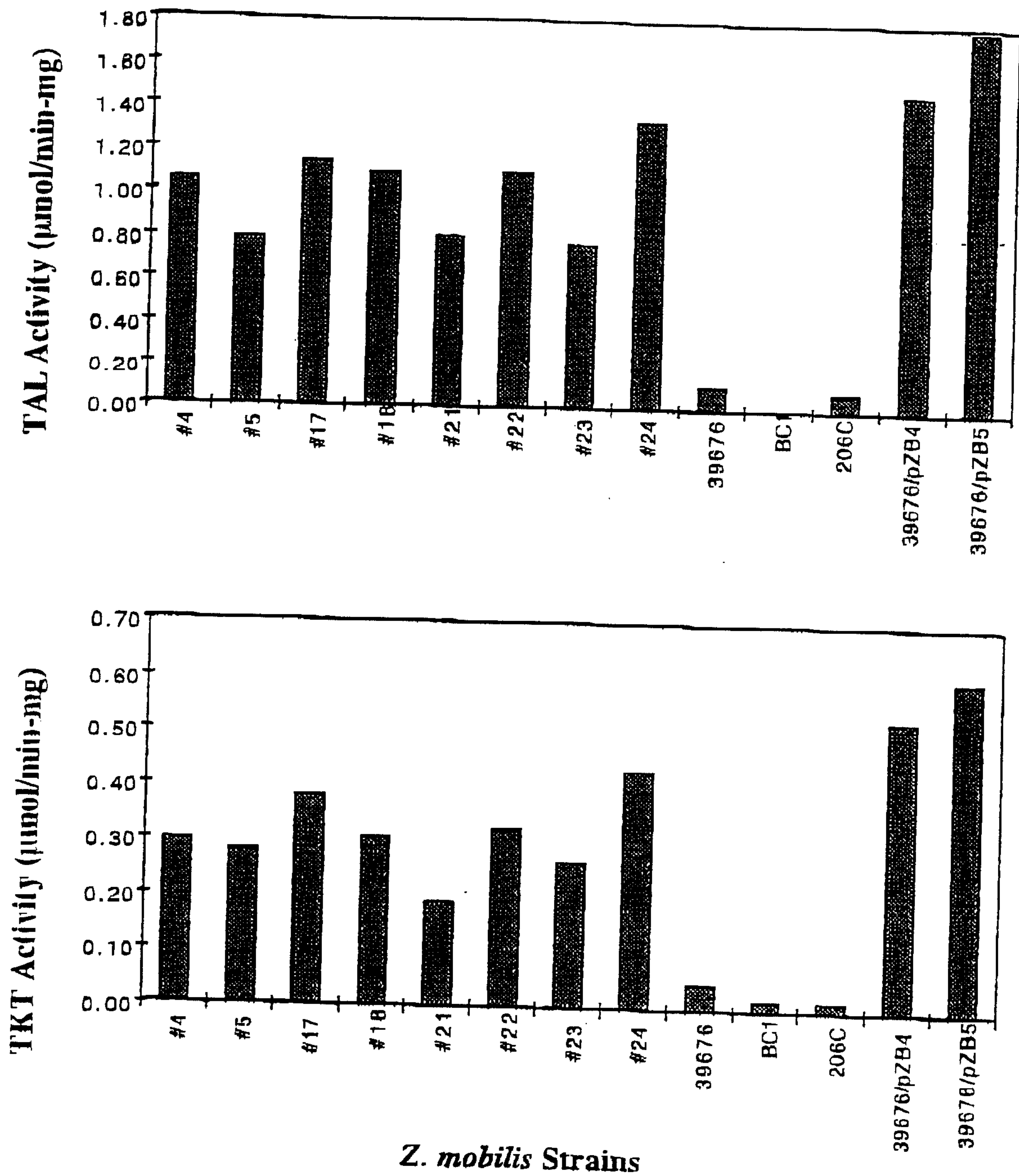
Feenayon & Singlehurst
 PATENT AGENTS



Southern analysis of tal/1kt-xy1Axy1B *Z. mobilis* transconjugates with Tnp probe.
C: control plasmid mini Tn 5 tal/1kt-xy1Axy1B. λ/H: DNA size marker.

FIG. 5

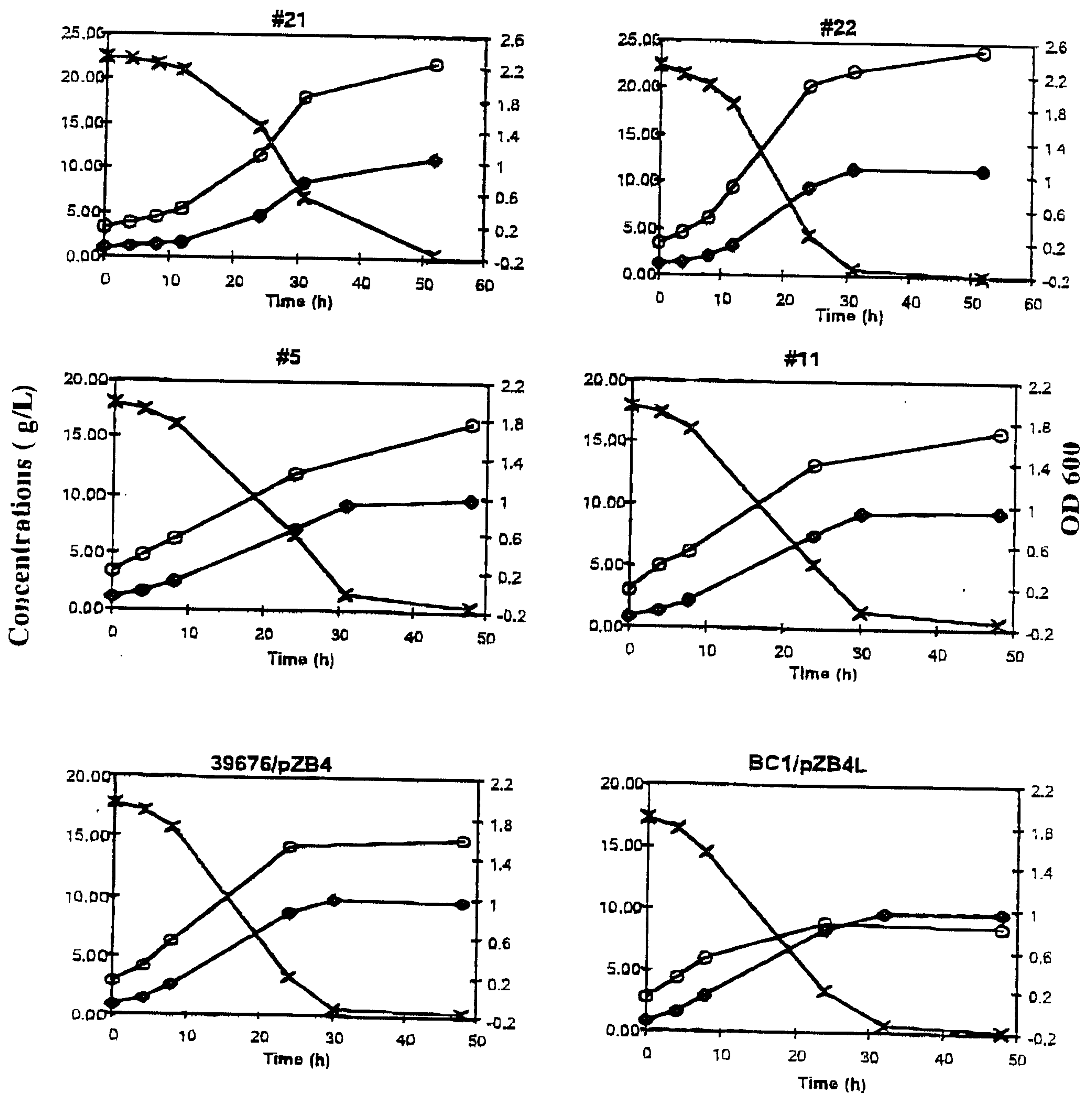
Fenderson & Seaglehurst
PATENT AGENTS



Enzymatic activities.

FIG. 6

Fealayan & Seaglehurst
PATENT AGENTS

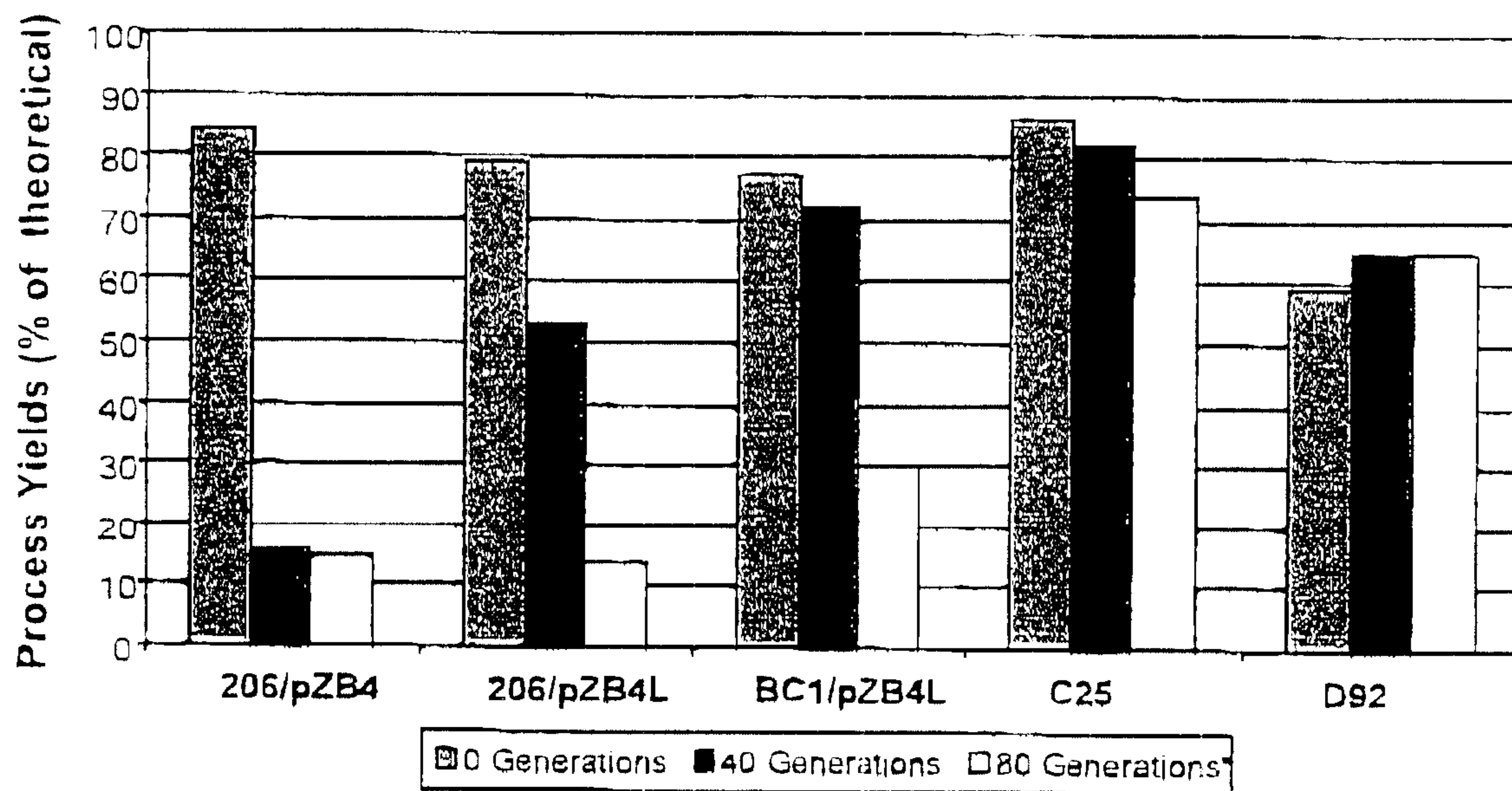


Fermentation profiles on RM+2%xylose
by *Z. mobilis* integrants and plasmid-bearing
Strains.

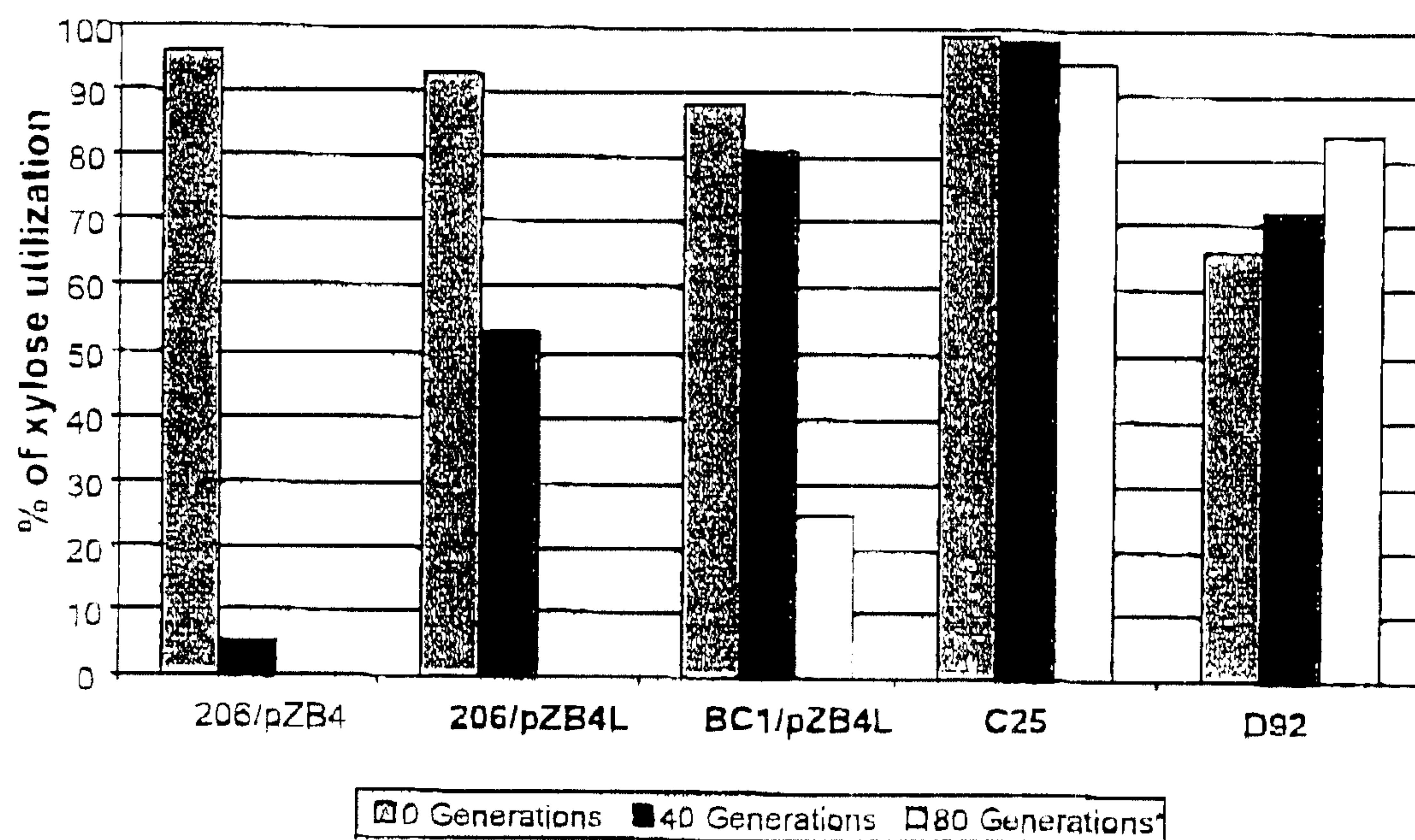
FIG. 7

Fealayon & Seiglerhurst
PATENT AGENTS

Process Yields

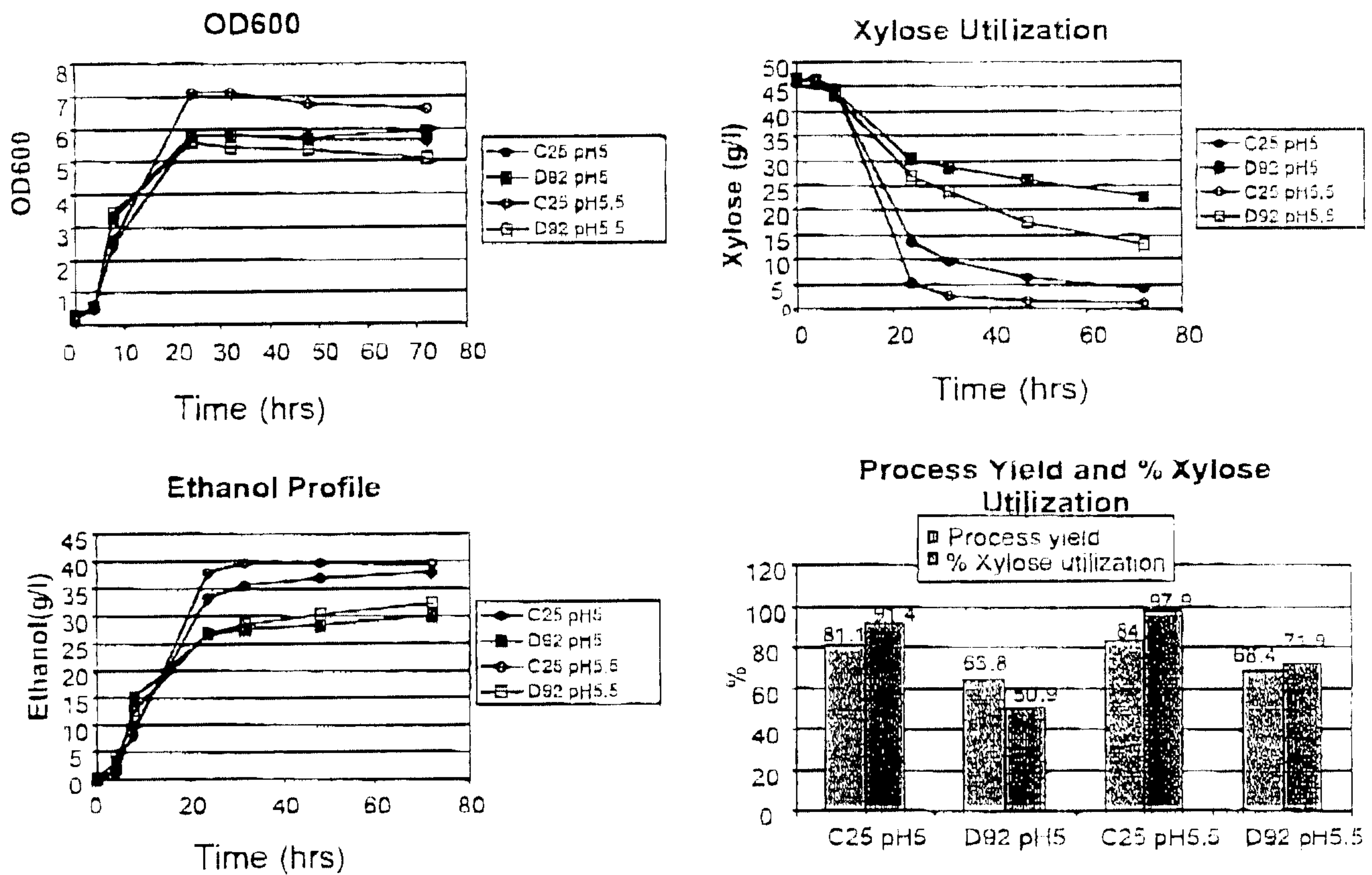


Xylose Utilization



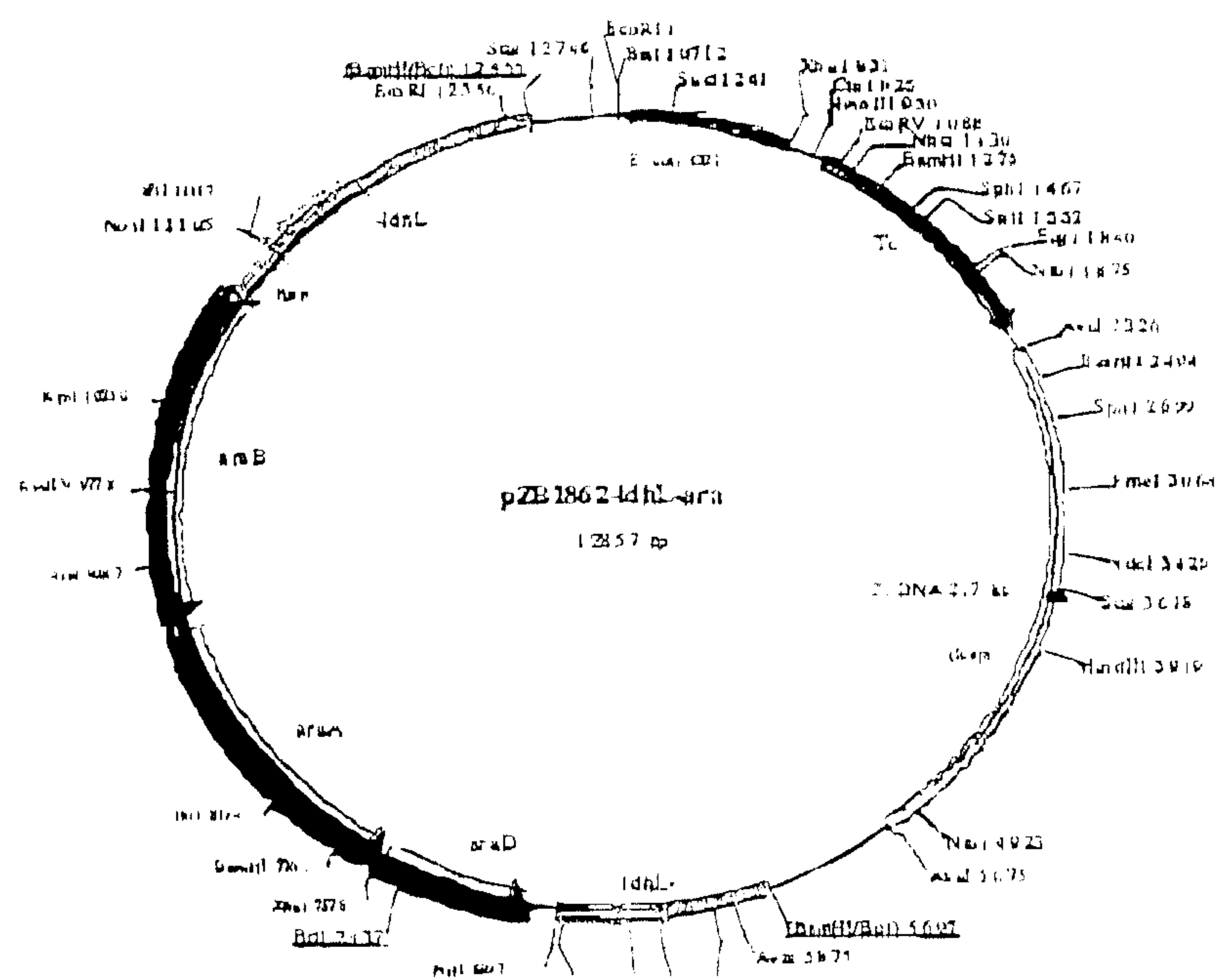
Stability of the plasmid-bearing and chromosomal integrated xylose-fermenting *Zymomonas* strains (* 90 generations for C25 and D95). The fermentation was conducted in flasks with RM containing 1% glucose and 5% xylose at 30°C without pH control.

FIG. 8



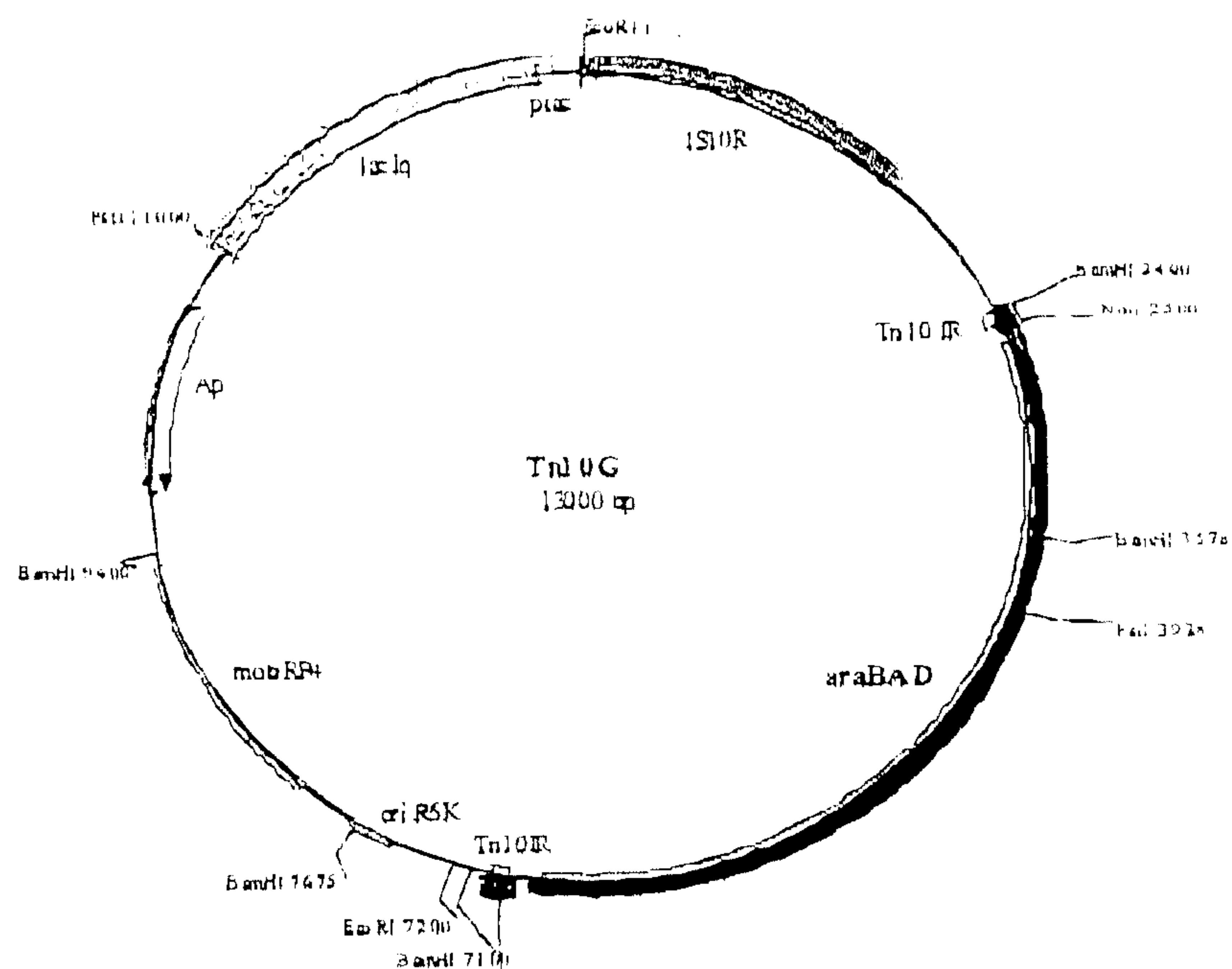
Batch fermentation of the chromosomal integrated xylose-fermenting *Zymomonas* strains, C25 and D95, in RM containing 4.5% glucose and 4.5% xylose at pH 5, pH5.5 and 30°C.

FIG. 9



Map of integrative plasmid pZB1862-ldhL-ara. The *araBAD* is inserted in the NotI site of *ldhL* disrupting *ldh*. The construction is based on a replicative plasmid pZB1862 of *Z. mobilis*.

FIG. 10



Plasmid map of Tn10G. IS10R: transposase gene. IR: inverted repeat. *araBAD* was inserted between two IRs at *NotI* sites.

FIG 11

Fig. 12A

Probe: ara
PstI digestion

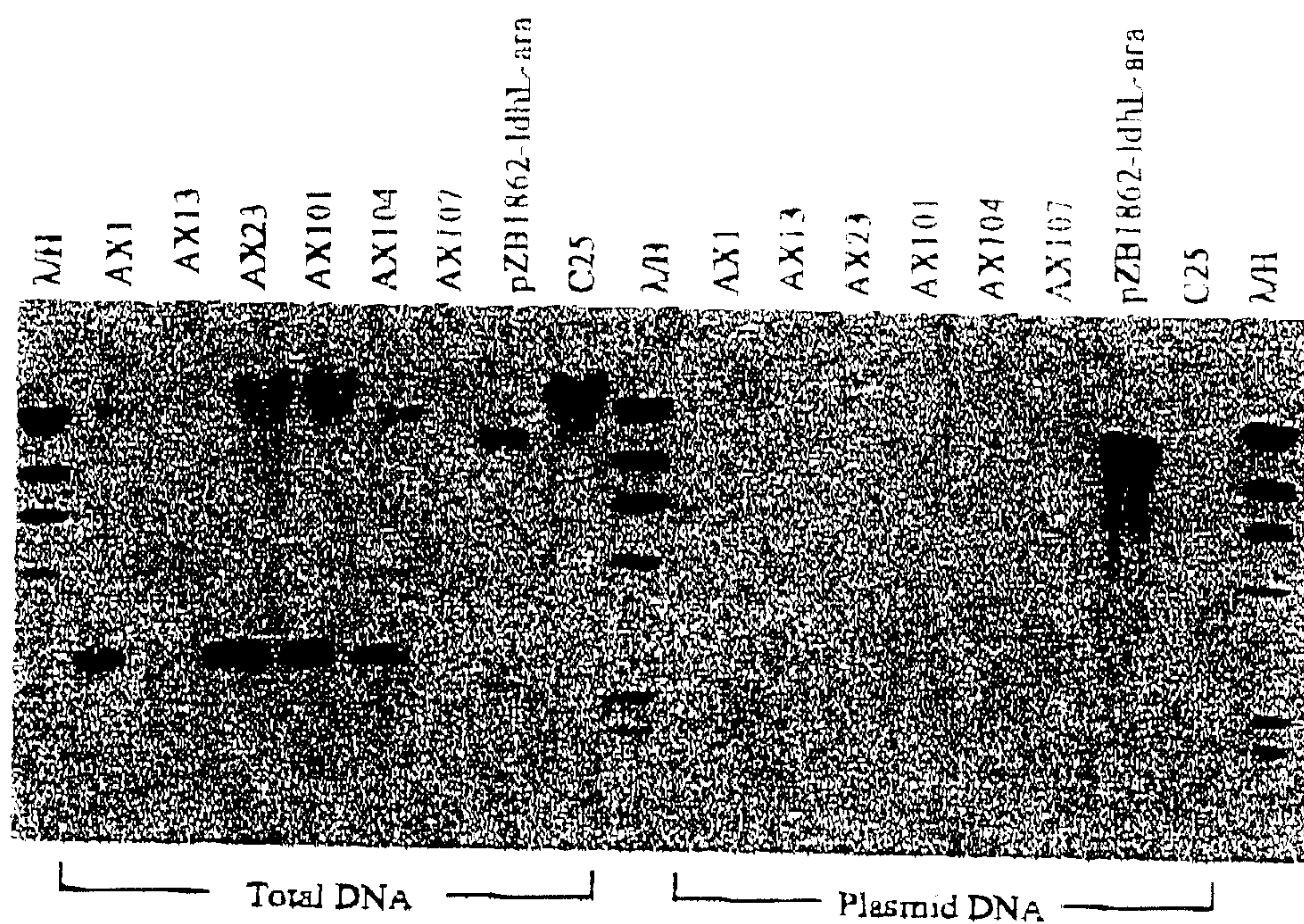
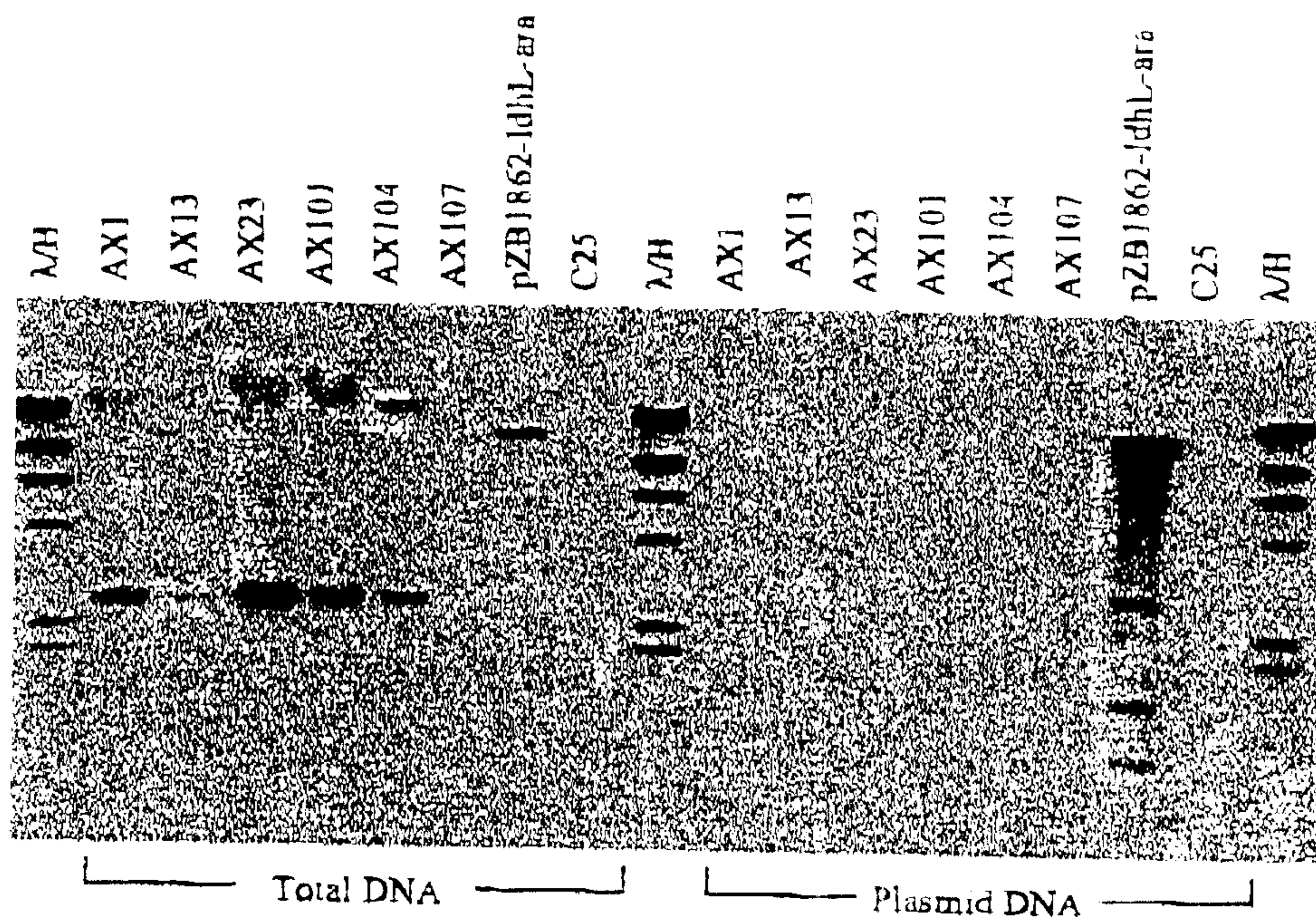


Fig. 12B

Probe: ldh
PstI digestion



Southern analysis of the chromosomal integrated xylose/arabinose-fermenting *Zymomonas* strains from homologous recombination using DIG-ara and DIG-ldh probes. AX1, 13, 23, 101, 104 and 107 are *araBAD* integrants. C25 is the host control. pZB1862-ldhL-ara is a plasmid control isolated from DH5α. λ/H is a molecular weight marker: 23, 9.4, 6.6, 4.3, 2.3 and 2.0 kb.

FIG. 12

Fig. 13B

Probe: mp
PstI digestion

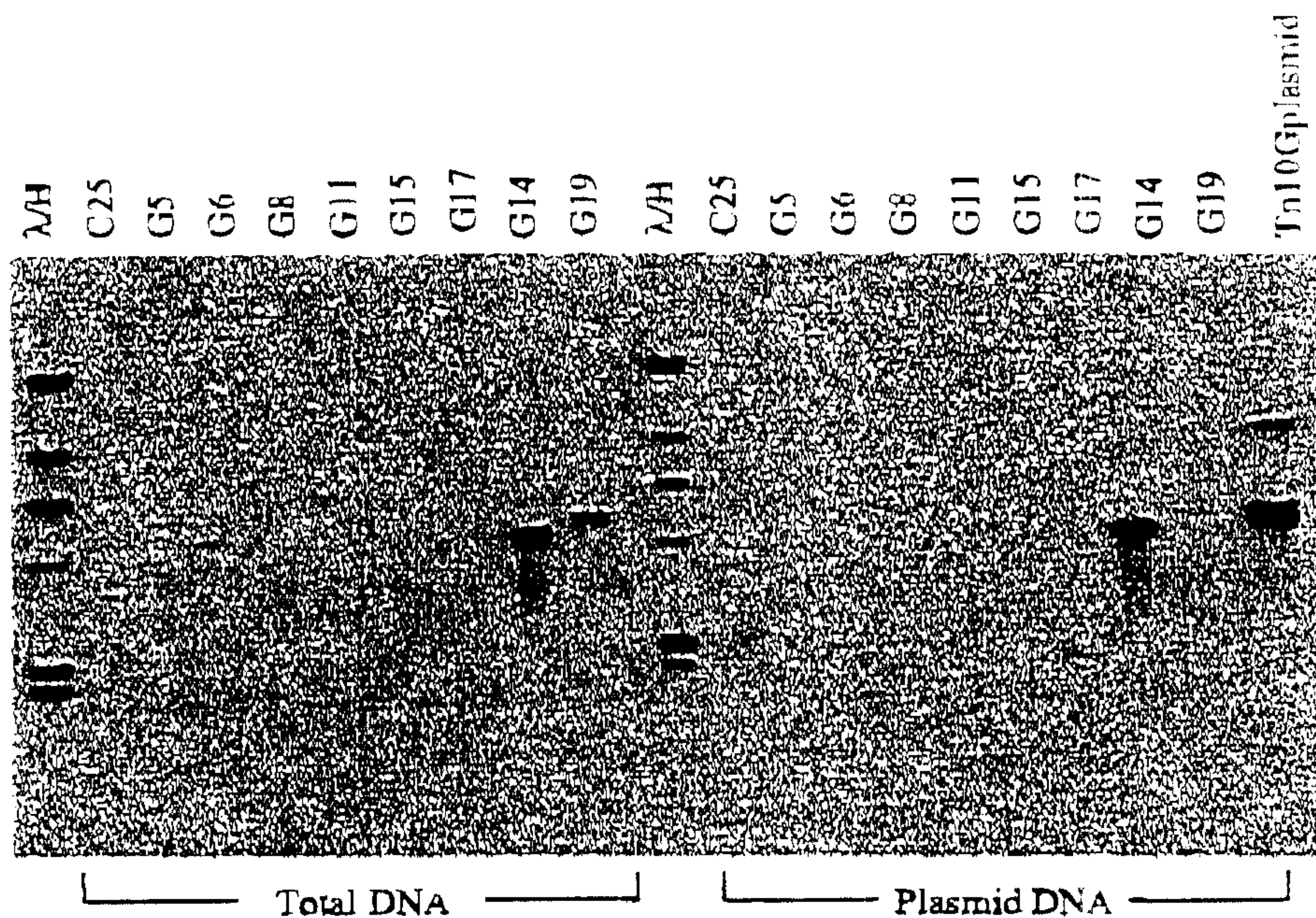
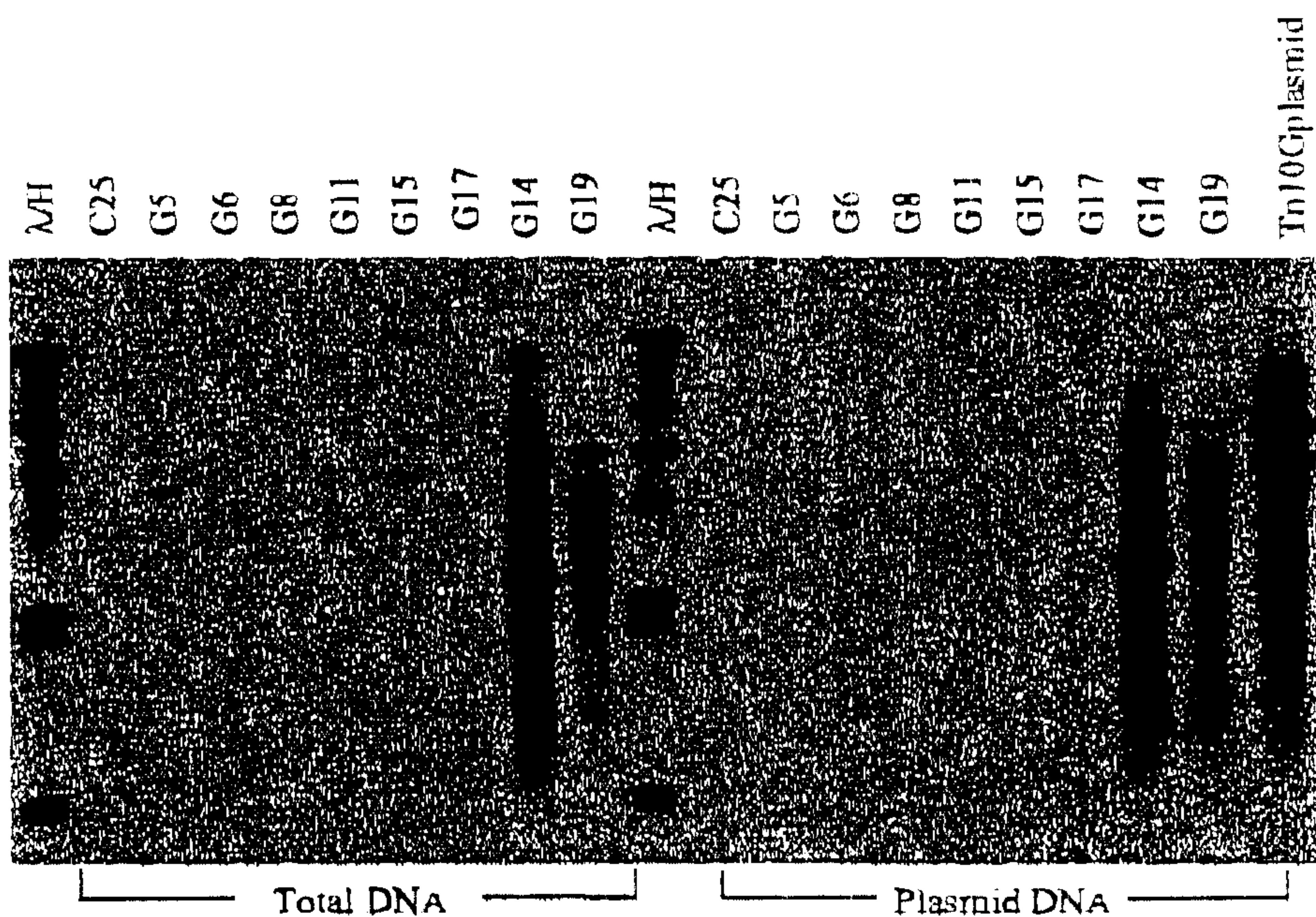


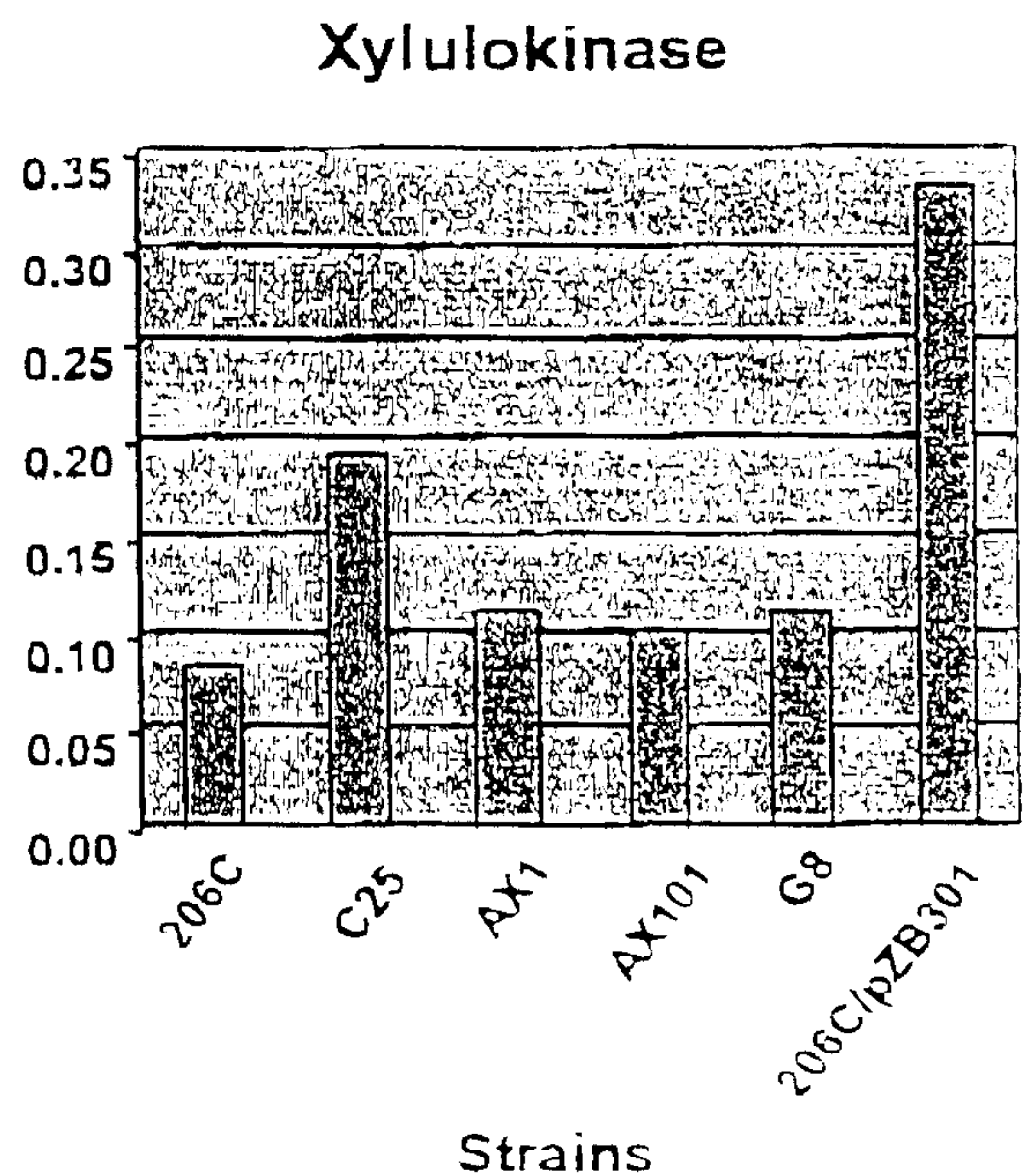
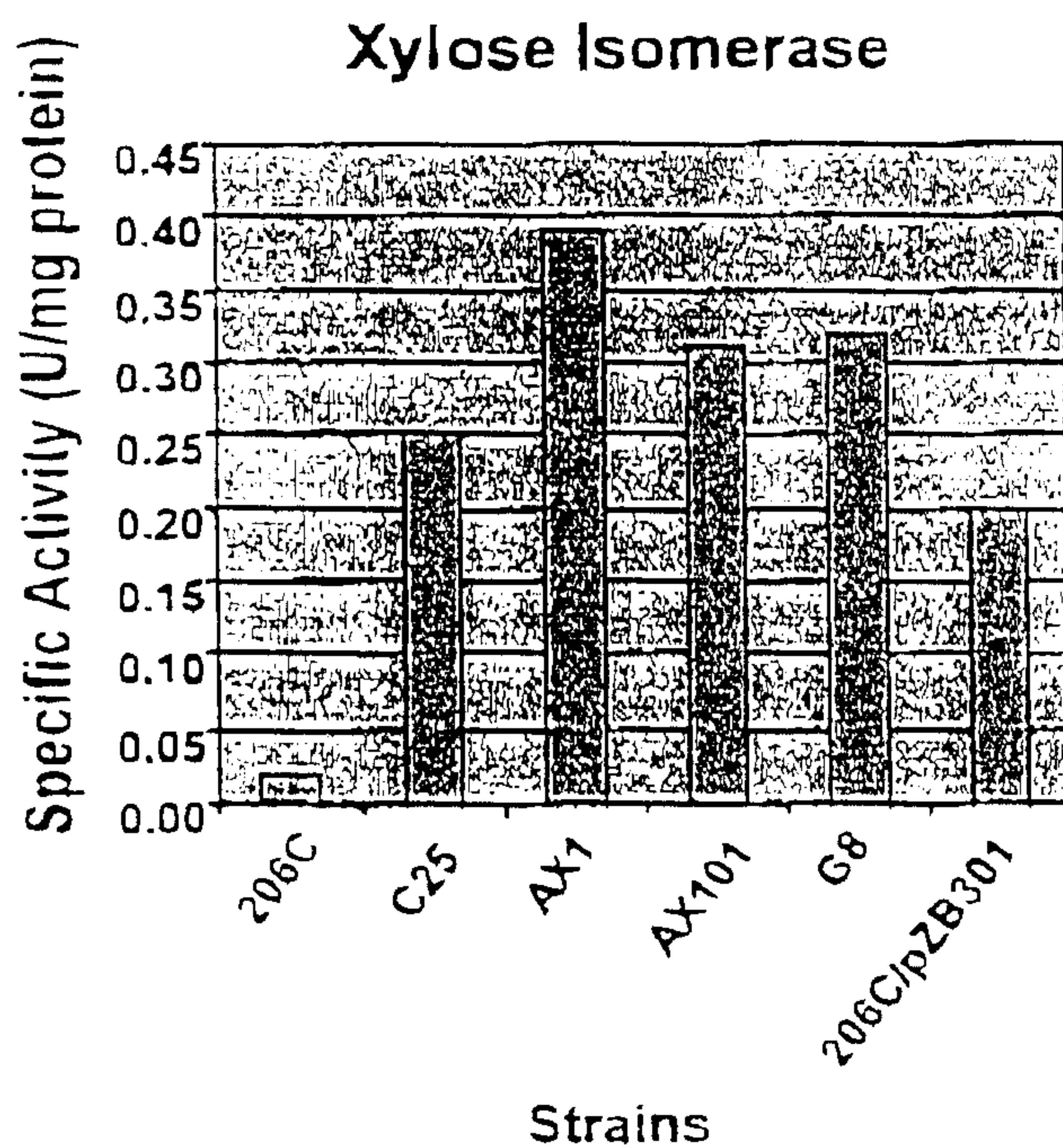
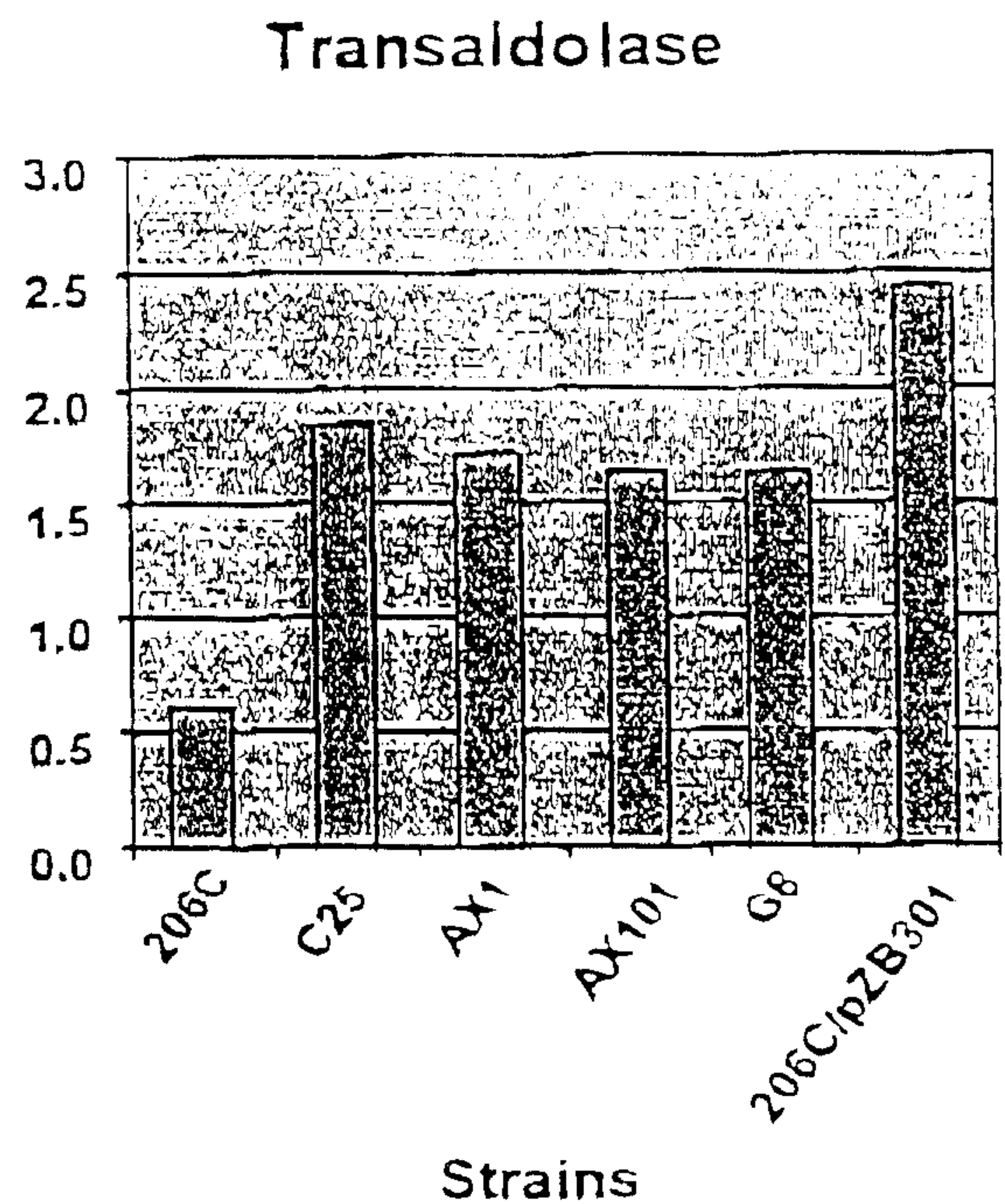
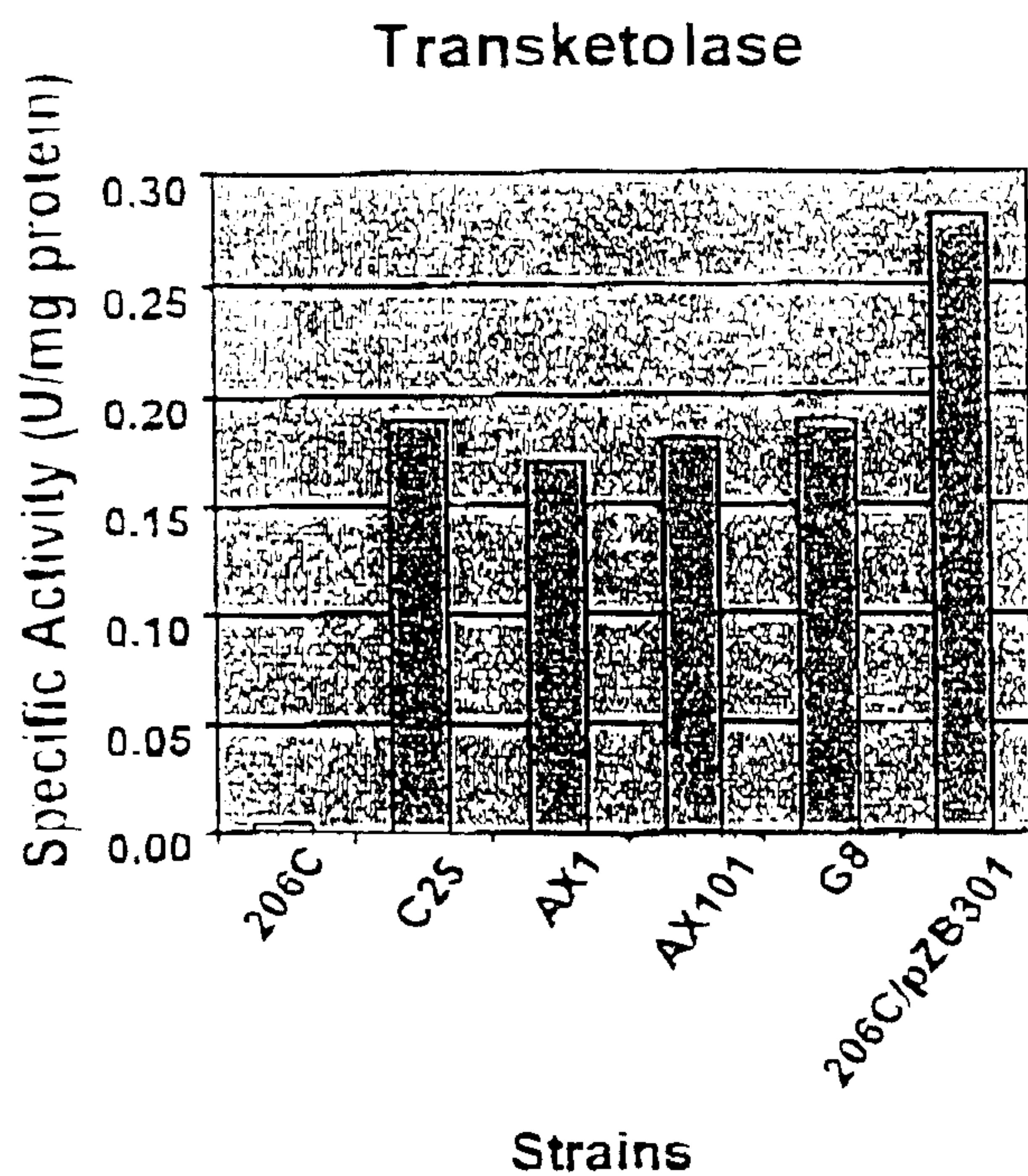
Fig. 13B

Probe: ara
PstI digestion



Southern analysis of the chromosomal integrated xylose/arabinose-fermenting *Zymomonas* strains from transposon integration using DIG-mp and DIG-ara probes. G5, 6, 11, 15, 17, 14 and 19 are *araBAD* integrants. C25 is the host control. Tn10G is the plasmid control. λH is a molecular weight marker: 23, 9.4, 6.6, 4.3, 2.3 and 2.0 kb.

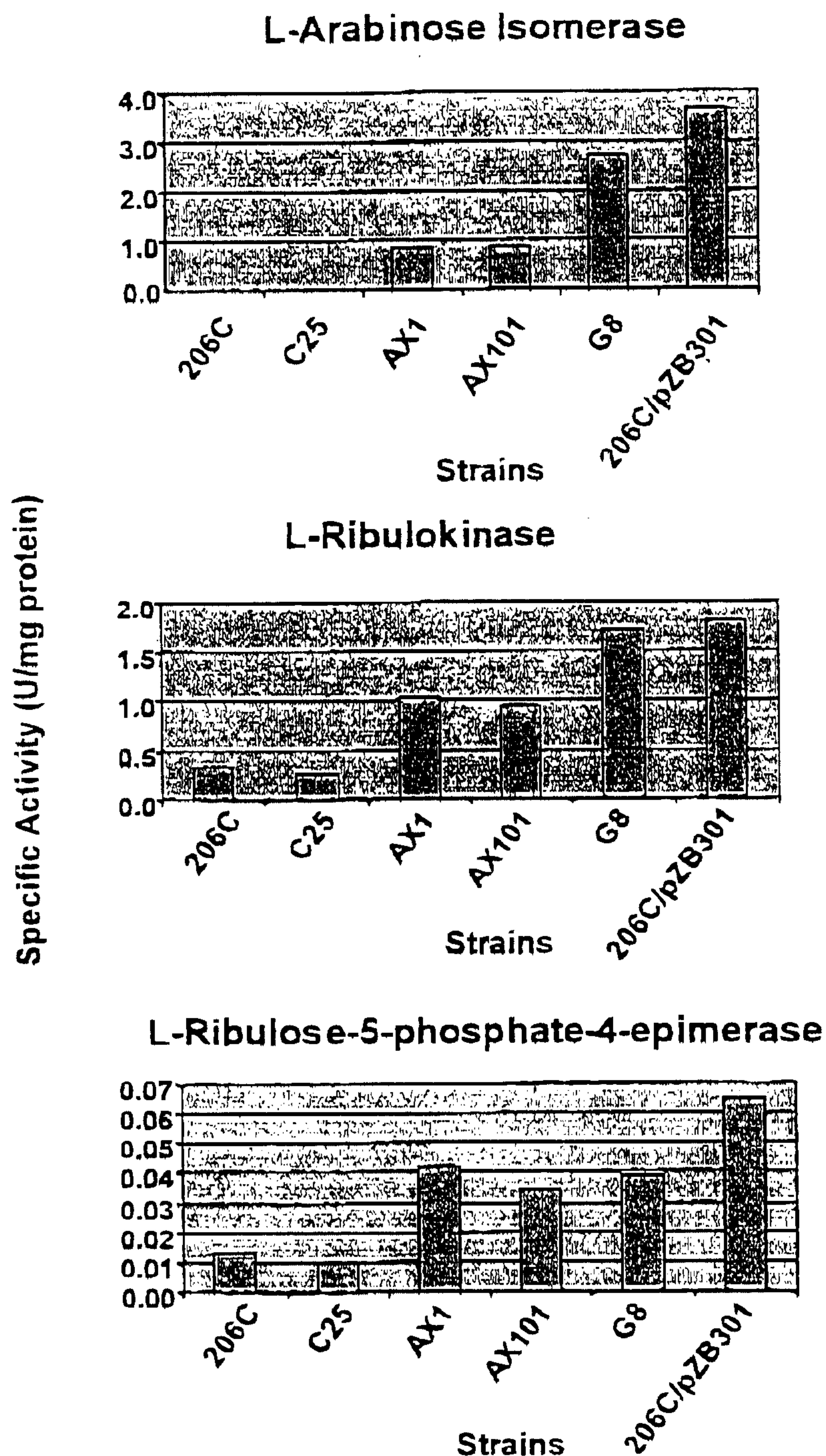
FIG. 13



Enzymatic activities of transketolase, transaldolase, xylose isomerase and xylulokinase of chromosomal integrated strains. 206C/pZB301 is a plasmid control. 206C is a host control. C25 is the xylose-fermenting integrant. G8 is the xylose/arabinose-fermenting integrant from Tn10 transposition. AX1 and AX101 are the xylose/arabinose-fermenting integrants from homologous recombination.

FIG. 14

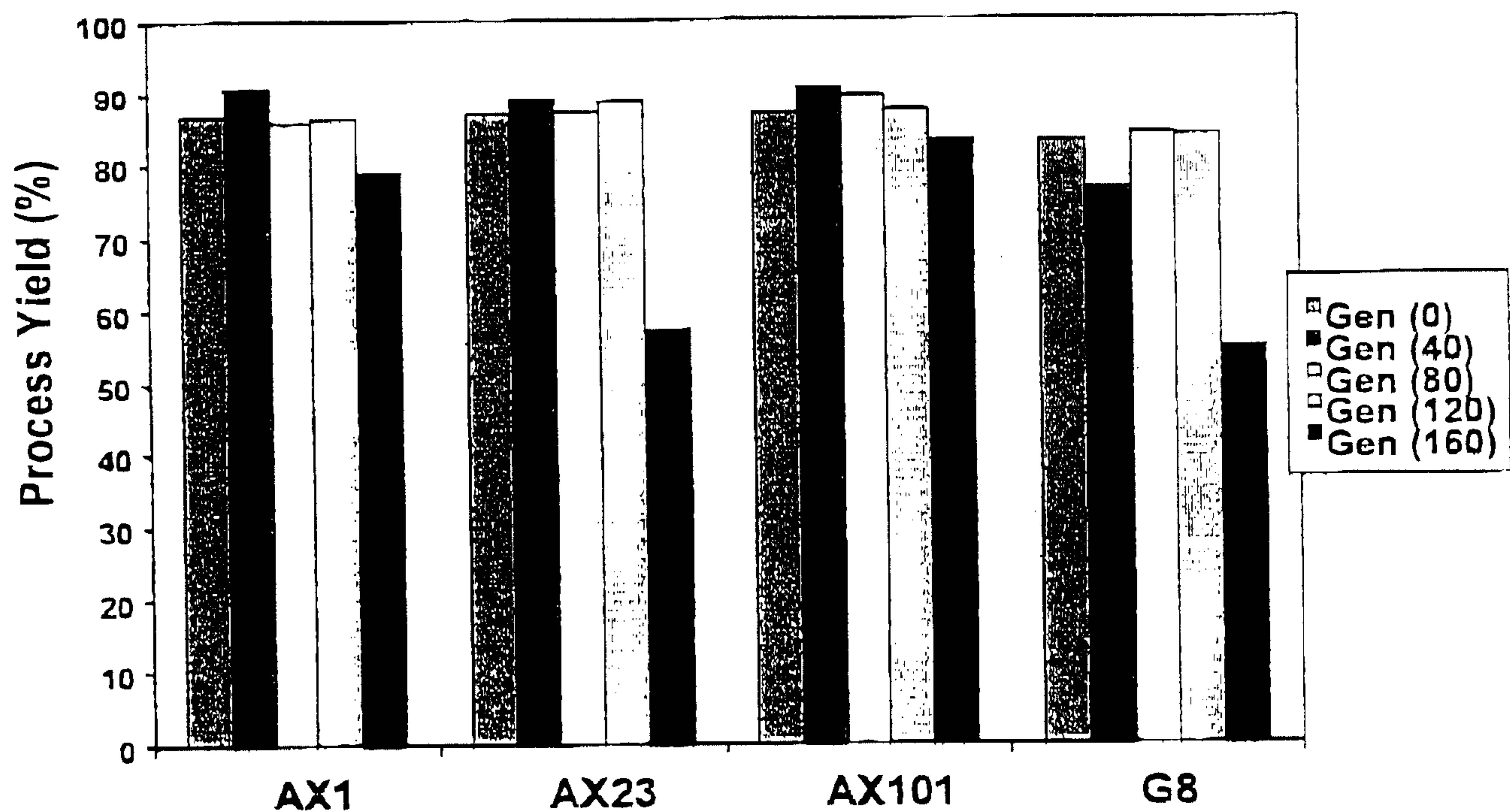
David James Hughes
PATENT AGENTS



Enzymatic activities of L-arabinose isomerase, L-ribulokinase and L-ribulose-5-phosphate-4-epimerase of chromosomal integrated strains. 206C/pZB301 is a plasmid control. 206C is a host control. C25 is the xylose-fermenting integrant. G8 is the xylose/arabinose-fermenting integrant from Tn10 transposition. AX1 and AX101 are the xylose/arabinose-fermenting integrants from homologous recombination.

FIG. 15

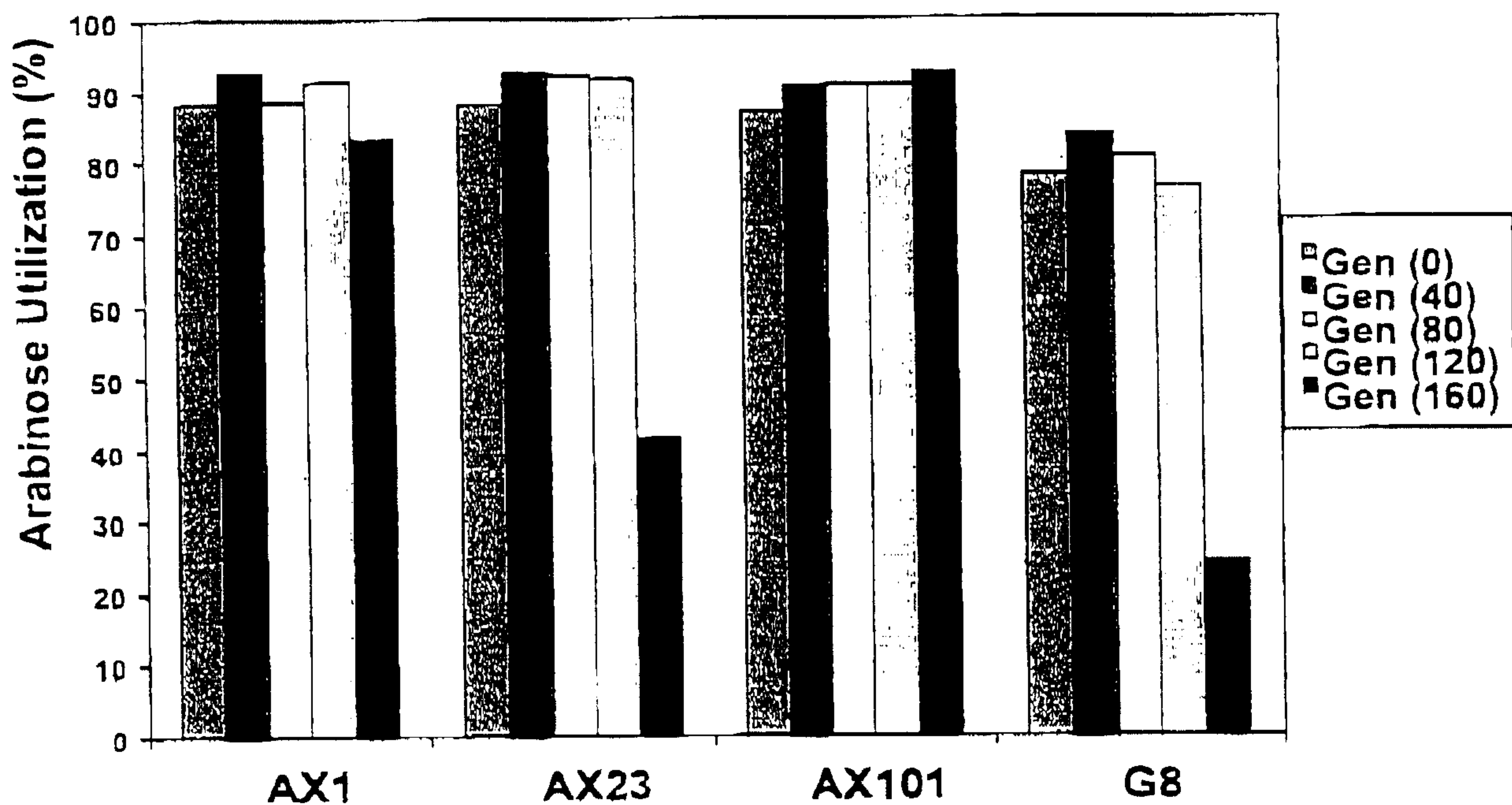
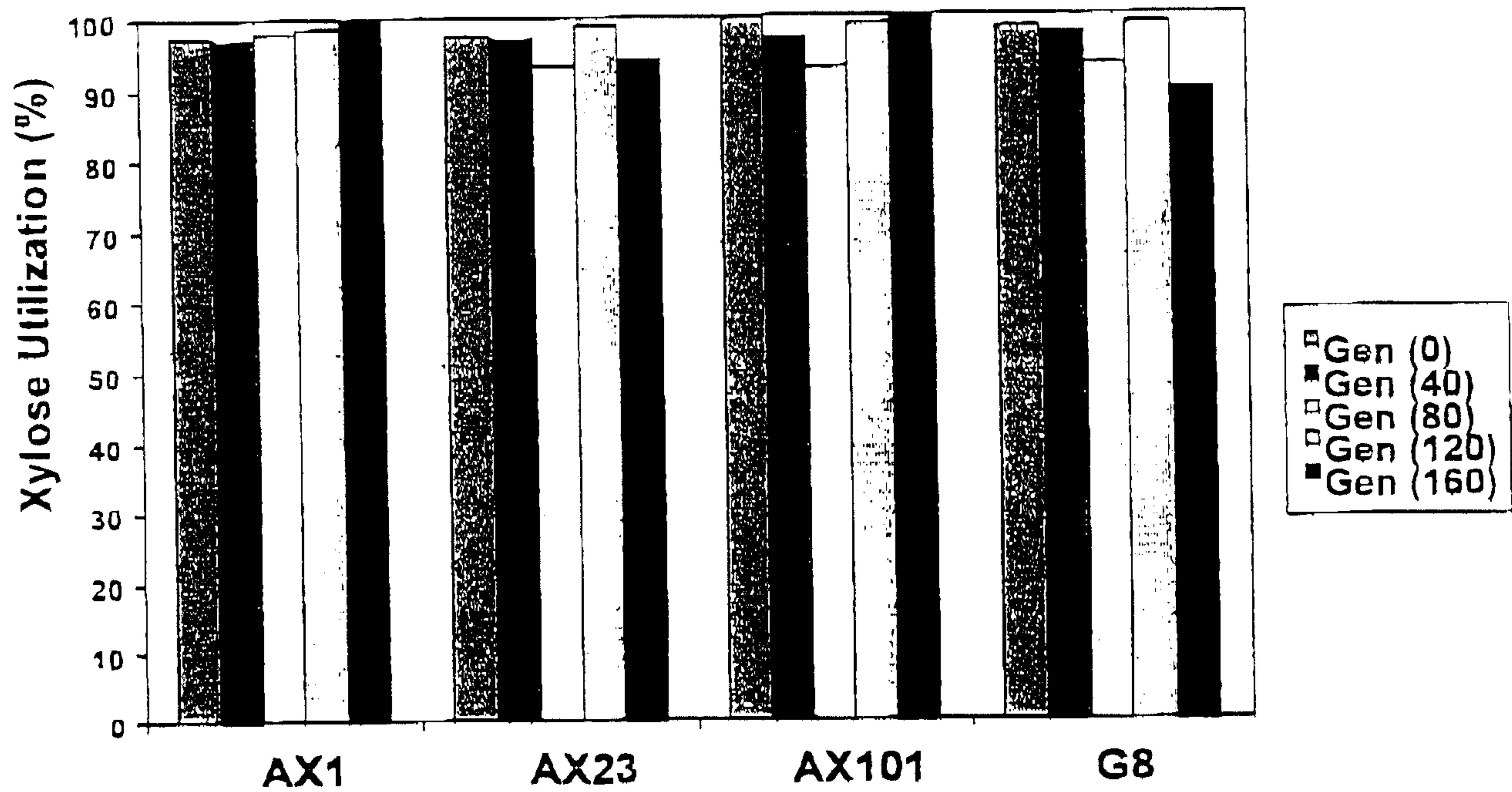
Tracy J. ...
PATENT AGENTS



Ethanol process yields of the chromosomal integrated xylose and arabinose-fermenting *Zymomonas* strains on RMGXA (1:2:2%) at $T=30^{\circ}\text{C}$ without pH control. These strains were inoculated from cultures at various generations on non-selective media.

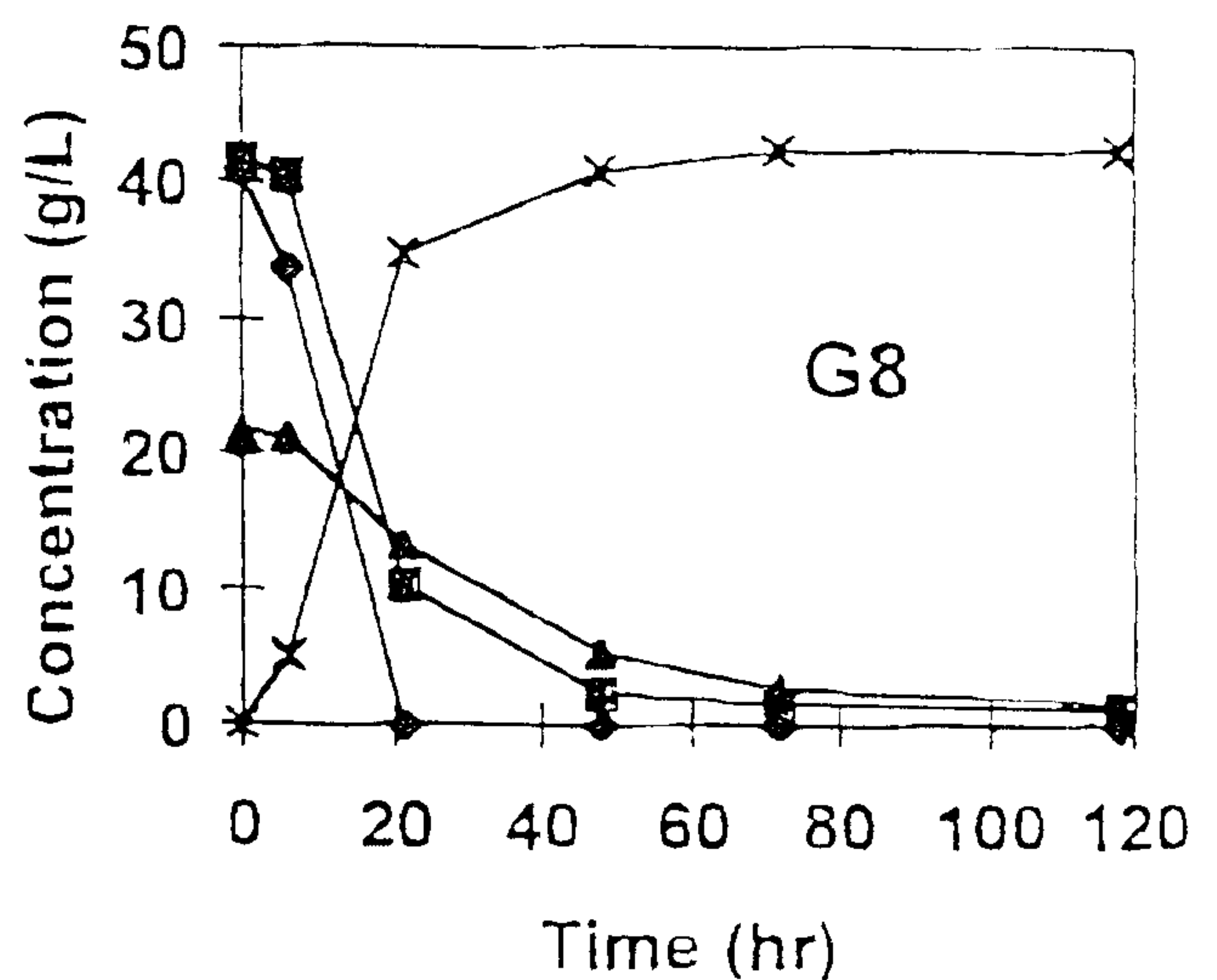
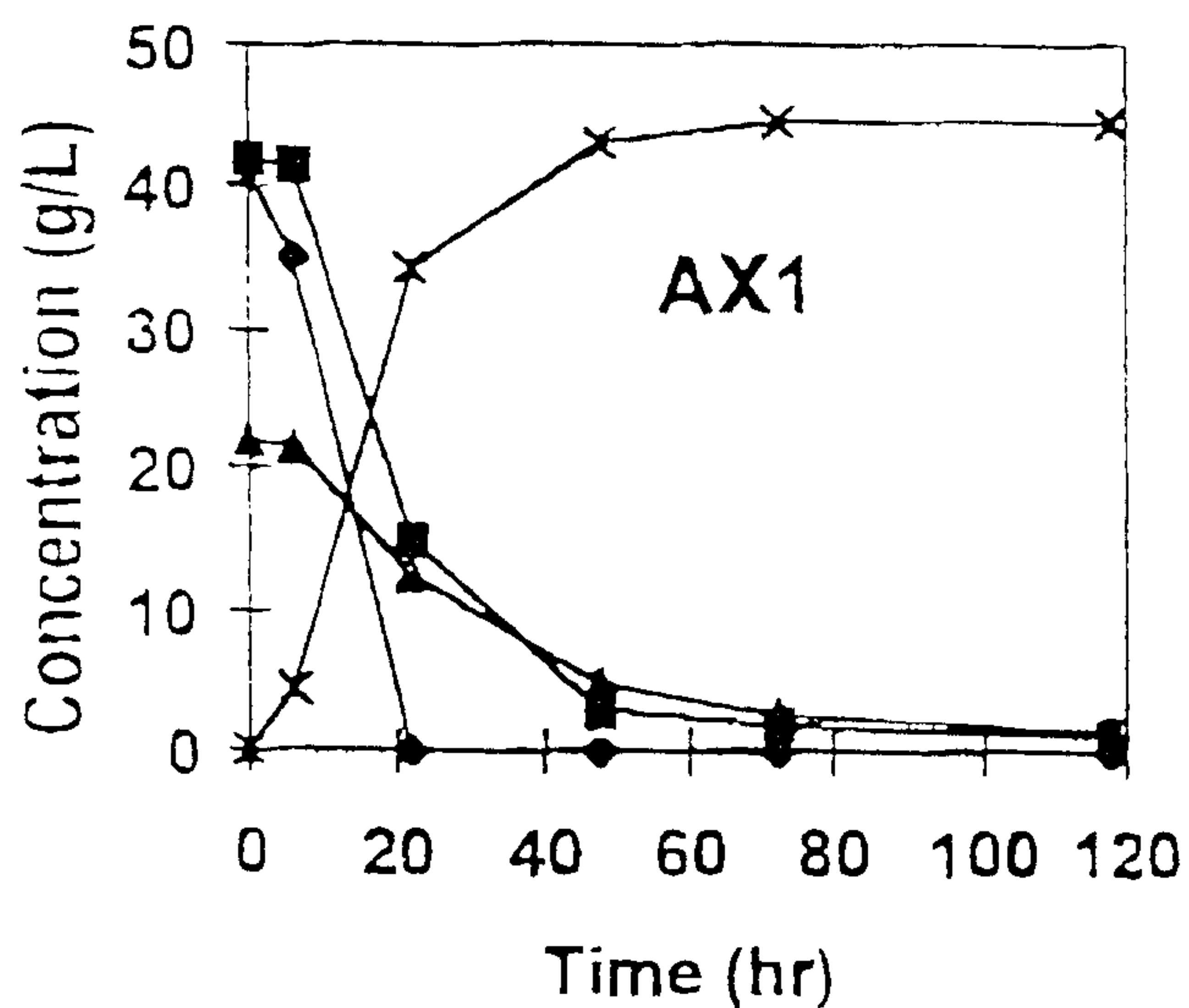
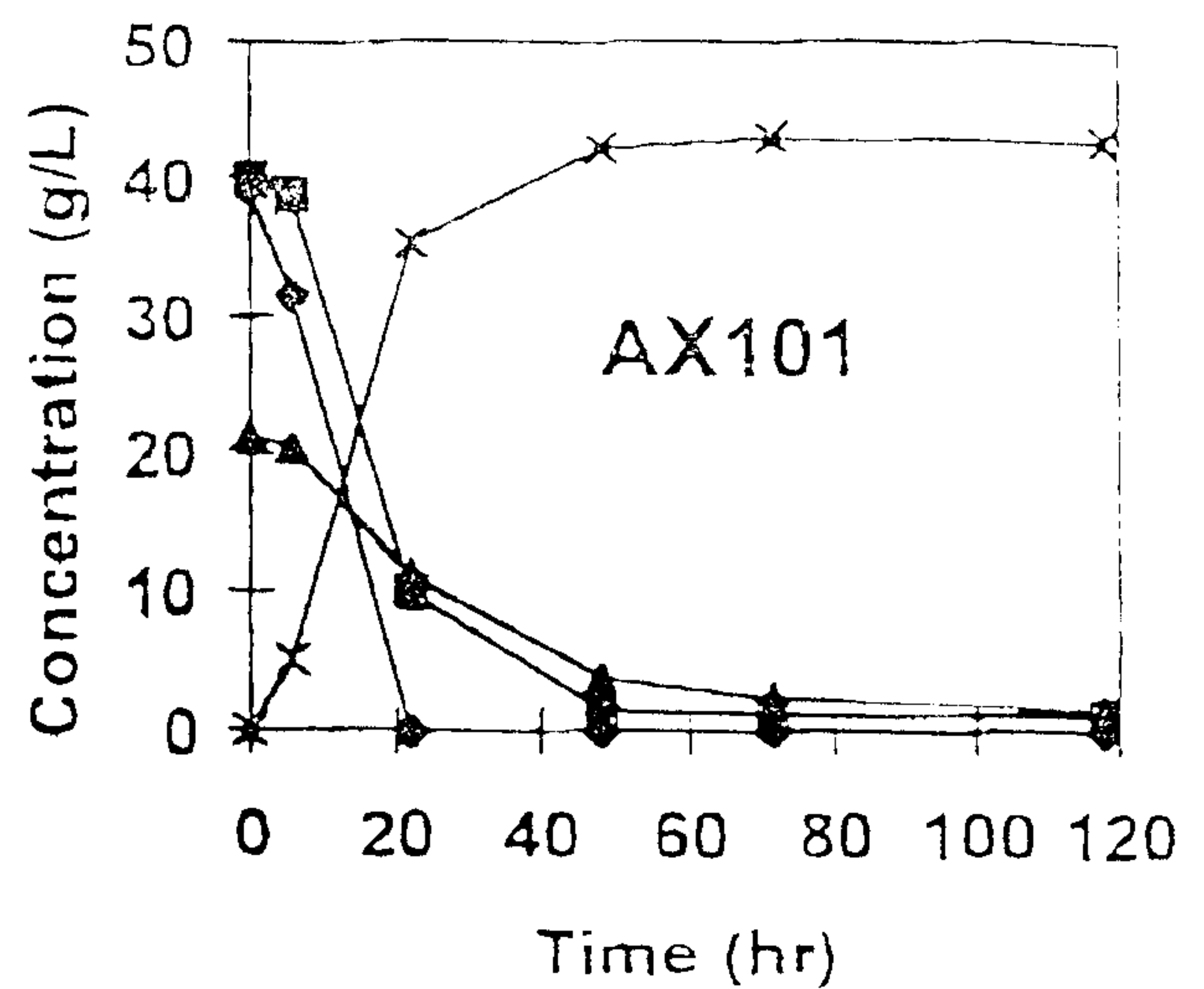
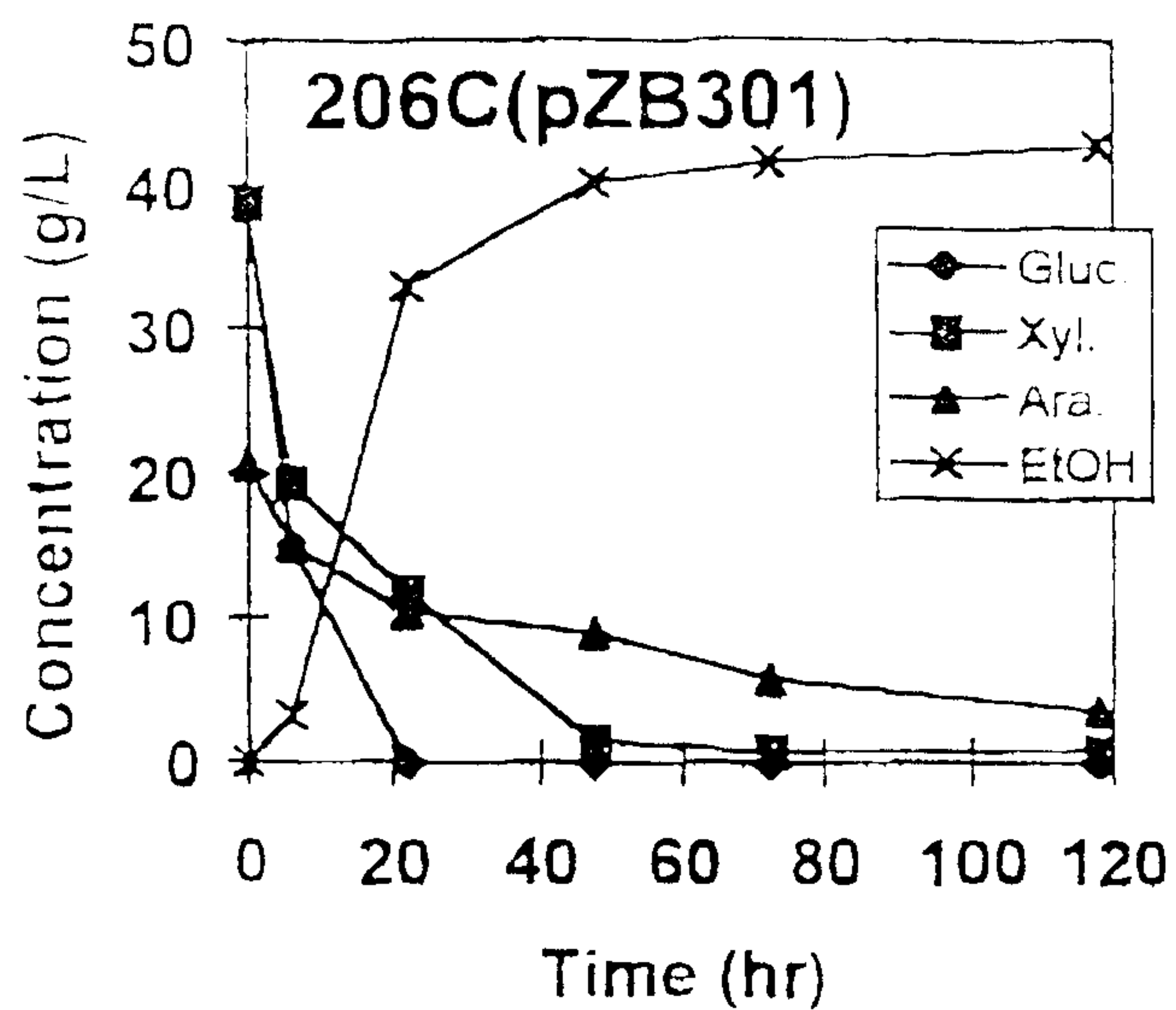
FIG. 16

PATENT AGENTS



Xylose and arabinose utilization of the chromosomal integrated xylose and arabinose-fermenting *Zymomonas* strains on RM containing 1% glucose, 2% xylose and 2% arabinose at 30°C without pH control. These strains were inoculated from cultures at various generations on non-selective media.

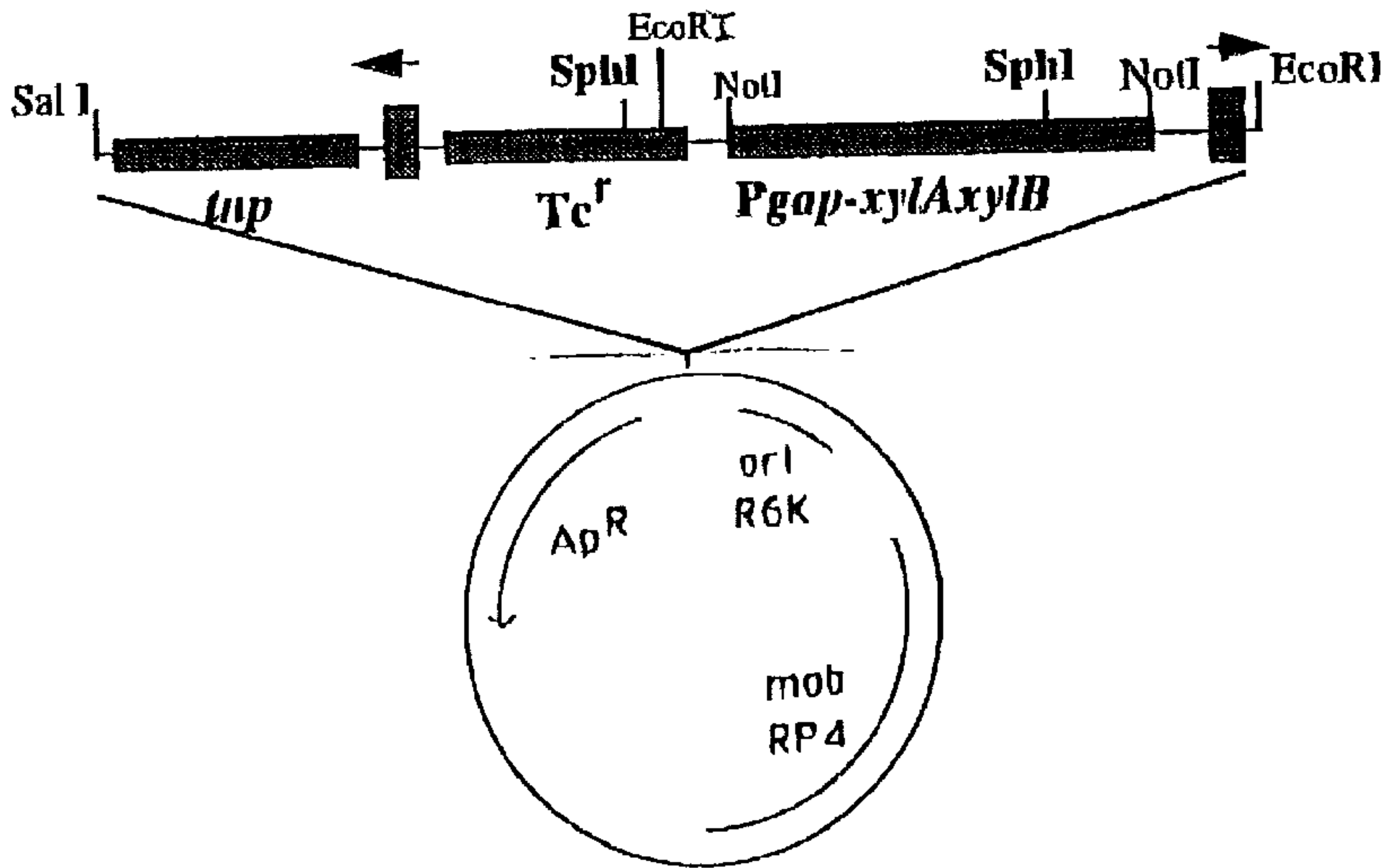
FIG. 17



Fermentation performance of chromosomal integrated xylose and arabinose-fermenting *Zymomonas* strains in RM containing 4% glucose, 4% xylose and 2% arabinose at pH 5.5 and 30°C.

FIG. 18

Faulstich & Fugleherst
PATENT AGENTS



Mini-Tn5 Tc with Xylose Assimilation Operon.