MICROBIOLOGY APPLICATIONS IN FOOD BIOTECHNOLOGY

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PREFACE

The Second Congress of the Singapore Society for Microbiology was held in Singapore in October 1989. One of the aims of the Congress Committee was to assemble leading scientists in food biotechnology for the purpose of obtaining a fruitful exchange of ideas in new developments in specific areas of food biotechnology. The SSM organised the Congress with the collaboration of the National University of Singapore, IUMS and *Trends in Biotechnology*.

The present volume consists of sixteen papers in food biotechnology. At a time when food biotechnology is making rapid developments in the improvement of production of enzymes, pigments, amino acids, terpenes and other substances deriving from microbial systems, we feel that a compilation of the papers in this volume should be welcomed.

The processes in food industries which use microbial enzymes such as lipase, amylases and proteases; molecular cloning genes for these enzymes in specific host organisms; the expression of these genes; the importance of systems for the processing of peptides and in the secretion of proteins form the topics of this volume. The development of molecular biological techniques for the improvement in the production of specific amino acids in Corynebacteria has been an important recent advance in food microbiology.

As in most other compilations on food science, food standards, food safety and food regulations and the methods of characterising microorganisms present in food are represented here.

We have, by design, selected specific topics for inclusion in this volume. It is our hope that the volume will meet the needs of food scientists and microbiologists in general.

NGA BEEN HEN
LEE YUAN KUN

CONTENTS

using the gene cloning technique, we constructed a new hybrid plasmid which had an insert of chromosomal DNA of B. pumilus coding the genes of xylanase (xynA) and 3-xylosidase (xynB). The expression of xynA and xynB were studied, including the DNA base sequence of the structural genes and promoter regions. For better understanding of xylanase function and to improve this enzyme by protein engineering, an X-ray crystallographic analysis of this enzyme was carried out at 2.2 A level resolution.

RESULTS

Cloning of xylanase and β-xylosidase genes of B.pumilus IPO into E.coli [2].

The ampicillin sensitive and tetracycline resistant clones of E.coli C600 transformed with the newly constructed hybrid plasmids, consisting of pBR322 and the chromosomal DNA fragments of B.pumilus were tested for their - xylosidase productivity. One clone among 439 was found to produce a yellow pigment by incubation of the cells with p-nitrophenyl-β-D-xyloside. The plasmid harbored in this clone was named pOXN29.

Isolated pOXN29 DNA was digested with various restriction enzymes and the resulting fragments were analyzed by agarose gel electrophoresis. From the results, the restriction map was obtained and is shown in Fig 1.

E.Coli harboring pOXN29 produced xylanase also, which was evidenced by the reducing sugar formation when a 1% xylan solution was incubated with the cell lyzate. Since neither the purified β-xylosidase not the cell extract of E.coli C600 can hydrolyze xylan, it was concluded that the above hydrolytic activity was caused by a xylanase gene encoded on pOXN29.

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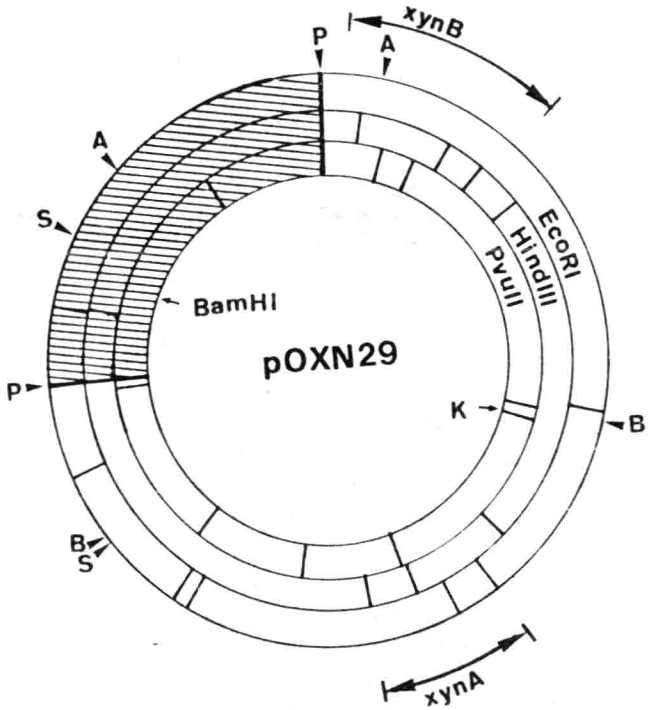

Figure 1. Restriction map of pOXN29. 13.0 of the PstI digested fragment of B.pumilus IPO chromosomal DNA (open area) was ligated at the PstI site of pBR322 (shadow area). A, B, K, P, S are respectively AvaI, Bg1II, KpnI, PstI and salI sites.

To determine the loci of the β-xylosidase and xylanse genes, smaller hybrid plasmids were derived from pOXN29 and are shown in Fig. 2. The pOXN29 gives two fragment of

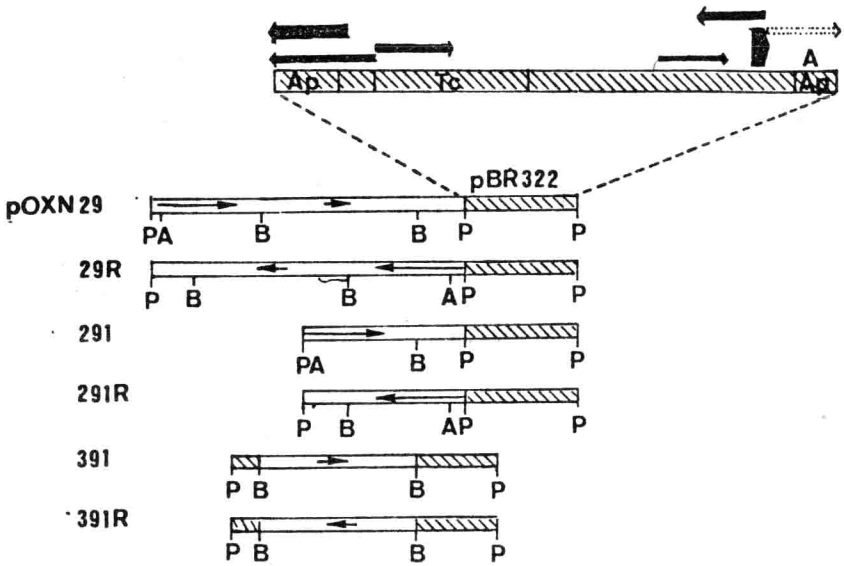

Fig.2 Relation between the structure of pOXN29 and its derivatives, and expression of β-xylosidase and xylanse in E.coli. The β-xylosidase productivity of pOXN29, 29R, 291, 291R, 391 and 391R are 0.92, 46.4, 1.0, 50.6, 0 and 0 respectively and that of xylanase of these plasmids are respectively 2.26, 9.59, 0, 0, 33.6 and 11.3.

11.4 and 6.3 kb by BglII digestion. The 11.3 kb fragment containing whole pBR322 DNA was ligated and transformed to E.coli C600. The transformants obtained were β-xylosidase positive and xlanase negative, indicating the locus of β-xylosidase within the 11.4 kb BglII fragment. This hybrid plasmid was named pOXN291. The 6.3 kb BglII fragment was subcloned into the BamHI sit of pBR322 and transformed into E.coli C600.

All transformants were xylanase positive and β-xylosidase negative indicating the locus of the xylanase

gene within the 6.3 kb fragment. Restriction analysis of the plasmid showed that both orientations of the 6.3 kb fragment result in xylanase production and they were named pOXN391 and 391R respectively.

To confirm the more precise location of both genes, pOXN291 and 391 were partially digested with HindIII or with EcoRI followed by ligation and transformation into E.coli C600. From the results, and the estimated gene size the location of the β-xylosidase gene on pOXN29 was restricted to between 0 and 3.8 kb, and that of xylanase gene to between 7.1 and 8.4 kb.

To obtain better expression of the genes under the control of the promoter of pBR322, which has been well studied [3], pOXN 29 and 291 were respectively digested with PstI followed by religation and transformation into E.coli 600. About half of the resulting plasmids were expected to have the foreign DNA in the opposite orientation, and were named pOXN29R and 291R. These increases in gene expression could be the effect of these structural gens, originally controlled by a weaker promoter, being transferred to a stronger promoter, that of 3-lactamase.

The location of xylanase and β-xylosidase as well as the marker enzymes are shown in Table 1. The location of xylanase, which is an extracellular enzyme in the donor cell, is in the cytoplasm in E.coli. More than 85% of the total activity of the marker enzymes was found in their respective fractions.

TABLE 1
Localization of xylanase and β-xylosidase in E.coli harboring the plasmid coding these enzyme genes

	Distribution of enzymes in fractions			
	Medium	Periplasm	Membrane	Cytoplasm
E.coli (pOXN29R) (osmotic shock)				
Xylanase	0	2	5	93
β-Xylosidase	-	4	4	92
β-Galactosidase	-	9	0	91
E.coli (pOXN29R) (lysozyme treatment)				
Xylanase	-	-	1	99
β-Xylosidase	-	-	0	100
β-Galactosidase	-	-	1	99
β-Lactamase	-	-	87	15

Subcloning of xylanase gene in B.subtilis [4].

A delation plasmid, pOXN293 (14.1 kb) harbored in the transformant which Ap^2, Tc^r, $xynA^+$, $xynB^+$ was found to have two EcoRI fragments deleted from pOXN29. Partial digest of pOXN293 with EcoRI site, and pOXW1 was recovered from E.coli transformants which were Tc^r, and Km^r, $xynA^+$, and $xynB^+$,.

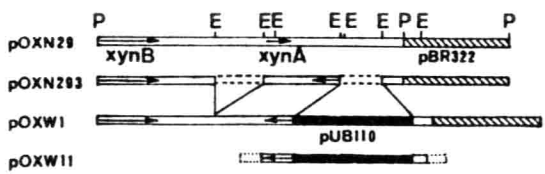

Fig 3. Structure of plasmids pOXYN293, pOXW1, pOXW11 and pOXW12

pOXW1 was introduced into B.subtillis was tested for the production of xylanase on a xylan-agar plate. The B.subtilis host excreted a small amount of xylanase that formed a clear zone around the colonies. About 50% of the regenerants of B.subtilis transformant cells formed larger clear zones than that of the host, and two plasmids pOXW11 and 12 were yielded from them. By analysis of the restriction fragments both plasmids were found to have delated DNA regions derived from pBR322 and a large part of B.pumilus chromosomal DNA including xynA but xynB was conserved. pOXW11 and 12 were stable maintained in their host cell in further cultures.

Table 2 shows the activities of xylanase and β-xylosidase synthesized by E.coli and B.subtilis cells harboring various plasmids. The E.coli host had no activity of β-xylosidase or xylanase, but the B.subtilis MI111 host had 3 times the β-xylosidase activity of the B.pumilus donor, and a little xylanase activity. β-Xylosidase was intracellular in all hosts and transformants. On the other hand, xylanse was excreted by Bacilli in the medium, but not by E.coli transformants. B.subtilis transformants excreted about 3 times as much xylanase as the donor strain, B.pumilus IPO.

Induction of xylanse and β-xylosidase [5].

Table 3 shows the production of xylanase and β-xylosidase by various clones in the presence of different sugars added to the L-broth. With B. pumilis both xylanase and β-xylosidase were induced strongly by xylose and partly by xylan and xylobiose. Both enzymes of B.subtilis were also induced by xylose. E.coli (pOXN29) synthesized similar amounts of both enzymes irrespective of the sugars added. B. subtilis harboring plasmids containing xynA also constitutively synthesized xylanase. These results suggest that gene(s) for the regulation of xylanase and β-xylosidase expression were not contained on the plasmids constructed.

TABLE 2
Activities of xylanase and β-xylosidase in various strains

	β-Xylosidase (m unit/mg protein)	Xylanase (m unit/mg protein)	
		Intra-cellular	Extra-cellular
B. pumilus IPO	16	0.39	600
E. coli C600	0	0	0
E. coli (pOXN29)	9.6	35	0
E. coli (pOXN293)	9.3	36	0
E. coli (pOXW1)	9.4	34	0
B. subtilis MI111	48	0.69	76
B. subtilis (pOXW11)	46	27	1,800
B. subtilis (pOXW12)	40	22	1,600

The nucleotide sequence of xyNA [6].

A deletion plasmid of pOXN391R, 392R, in which the 3.2 kb HindIII fragment was delated from pOXN391R was constructed by partial digestion with HindIII and religation. The nucleotide sequence from the EcoRI site at 2.1 kb to the HindIII site at 3.2 kb was determined. The nucleotide sequence of 1070 bp covering the entire xynA gene and its flanking regions is shown in Fig. 4.

TABLE 3
Induction of xylanase and β-xylosidase

Sugar added	β-Xylosidase (m unit/mg protein)			
	B.pumilis IPO	B.subtilis MI111	E.coli (pOXN29)	B. subtilis (pOXW11)
None	0.1	1	2.3	
Glycerol	0.2	1	2.0	
Glucose	0.1	1	2.9	
Xylose	16.0	31	4.0	
	Xylanase (m unit/mg protein)			
None	1	17	23	1990
Glycerol	2	16	29	1910
Glucose	1	21	33	1880
Xylose	600	76	35	1800

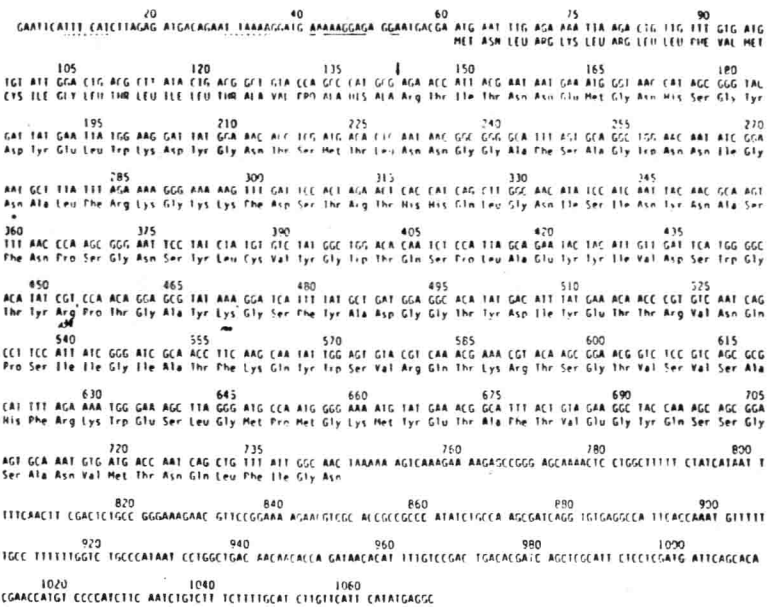

Fig 4. The nucleotide sequence of xynA and the amino acid sequence of xylanase deduced from it.

The sequence is consistent with the observed restriction fragments. Of three reading frames found, one was 684 bp open reading frame beginning at 61 bp. The amino sequence of the N-terminal region of B. pumilus xylanase, which is excreted in the culture medium, was determined to be Arg-Thr-Ile-Thr- by sequential Edman degradation followed by identification of the PTH-amino acid. This finding suggests that the signal peptide consisting of 27 amino acid residues is processed between Ala^{27} and Arg^{28}. The processed xylanase was deduced to consist of 201 amino acid residues, corresponding to an Mr of 22,384, which agrees with the Mr of purified xylanase of B. pumilus estimated by equilibrium ultracentrifugation and SDS-polyacrylamide gel electrophoresis. The ribosome binding sequence complementary to the 3' -end of 16S rRNA of B.subtilis, 3'-UCUUUCCUCCACUAG-5' was observed 7 bp upstream of the initiation codon, ATG.

Expression of the xylanase gene in Saccharomyces cerevisiae [7]

To bread a yeast strain capable to produce xylanase, pNAX2 was designed to code for the mature xylanase gene added with Met at its N-terminus as the translational initiation site just downstream to the EcoRI site of the GAP (the glycer-aldehyde-3-phosphate dehydorgenase gene) promoter (Fig.5).