

MICROBIOLOGY
APPLICATIONS
IN FOOD
BIOTECHNOLOGY

MICROBIOLOGY APPLICATIONS IN FOOD BIOTECHNOLOGY

Edited by

B.H. NGA and Y.K. LEE

National University of Singapore, Kent Ridge, Singapore



ELSEVIER APPLIED SCIENCE
LONDON and NEW YORK

ELSEVIER SCIENCE PUBLISHERS LTD
Crown House, Linton Road, Barking, Essex IG11 8JU, England

Sole Distributor in the USA and Canada
ELSEVIER SCIENCE PUBLISHING CO., INC.
655 Avenue of the Americas, New York, NY 10010, USA

WITH 37 TABLES AND 33 ILLUSTRATIONS

© 1990 ELSEVIER SCIENCE PUBLISHERS LTD

British Library Cataloguing in Publication Data

Microbiology applications in food biotechnology.

1. Food technology. Applications of biotechnology

I. Nga, B. H. II. Lee, Y. K.

664

ISBN 1-85166-530-7

Library of Congress CIP data applied for

No responsibility is assumed by the Publisher for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions or ideas contained in the material herein.

Special regulations for readers in the USA

This publication has been registered with the Copyright Clearance Center Inc. (CCC), Salem, Massachusetts. Information can be obtained from the CCC about conditions under which photocopies of parts of this publication may be made in the USA. All other copyright questions, including photocopying outside the USA, should be referred to the publisher.

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, recording, or otherwise, without the prior written permission of the publisher.

Printed in Great Britain by The Alden Press, Oxford

MICROBIOLOGY APPLICATIONS IN FOOD BIOTECHNOLOGY

Proceedings of the Second Congress of the Singapore Society for Microbiology, Singapore, 31 October-3 November 1989.

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

<i>Preface</i>	v
Expression of the xylanase gene of <i>Bacillus pumilus</i> in <i>Escherichia coli</i> , <i>B. subtilis</i> and <i>Saccharomyces cerevisiae</i>	1
<i>H. Okada</i>	
Chromosome engineering in <i>Saccharomyces cerevisiae</i> by using a site-specific recombinant system of a yeast plasmid	13
<i>Y. Oshima</i>	
Molecular genetics of <i>Corynebacteria</i> : cloning and characterization of the tryptophan operon and the genes of the threonine biosynthetic pathway	20
<i>J.F. Martin, L.M. Mateos, R.F. Cadenas, C. Guerrero, M. Malumbres, A. Colinas and J.A. Gil</i>	
Cloning of the alkaline extracellular protease gene of <i>Yarrowia lipolytica</i> and its use to express foreign genes	27
<i>H. Heslot, J.M. Nicaud, E. Fabre, J.M. Beckerich, P. Fournier and C. Gaillardin</i>	
Mitotic segregation in intergeneric hybrids of yeast to give novel genetic segregants	46
<i>B.N. Nga, T.L.S. Chee, H.Y. Tan and F.D. bte Abu Bakar</i>	
Genetic and technological improvements with respect to mass cultivation of microalgae.	61
<i>Y.K. Lee</i>	
Biotechnology of enzymes and pigments	74
<i>A.L. Demain, T. Kobayashi, T.F. Lin, S. Nochur, M.P.M. Romaniec and M.F. Roberts</i>	
* Hydration of nitriles to amides by microbial enzymes	88
<i>H. Yamada and T. Nagasawa</i>	

Production of monoterpenes by yeast mutants defective in sterol biosynthesis	101
<i>C. Javelot, F. Karst, V. Ladeveze, C. Chambon and B. Vladescu</i>	
Drugs from the sea	123
<i>S. Halevy</i>	
Role of intestinal flora in health with special reference to dietary control of intestinal flora	135
<i>T. Mitsuoka</i>	
Food regulations and food safety	149
<i>J.H.B. Christian</i>	
Microbiological criteria in regulatory standards: reason or rhetoric. . . .	162
<i>R.G. Bell and C.O. Gill</i>	
Safety assessment of genetic manipulation of microorganisms and plants, as applied to foods	177
<i>J.E. Smith</i>	
<i>Salmonella</i> , the organism, its occurrence and prevention in foods	189
<i>W. Budnik</i>	
The application of DNA probes in food microbiology	219
<i>K.H. Schleifer</i>	
<i>Index of Contributors</i>	232

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 Å 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.

E.coli C600 can hydrolyze xylan, it was concluded that the above hydrolytic activity was caused by a xylanase gene encoded on pOXN29.

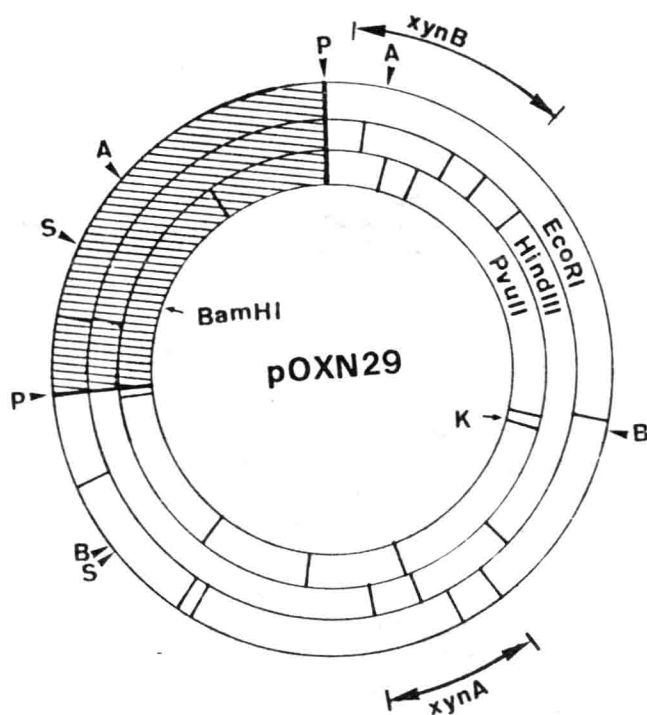


Figure 1. Restriction map of pOXN29. 13.0 of the PstI digested fragment of B.pumilus IPO chromosomal DNA (open area) was ligated at the PstI site of pBR322 (shadow area). A, B, K, P, S are respectively AvaI, BglII, KpnI, PstI and SalI sites.

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

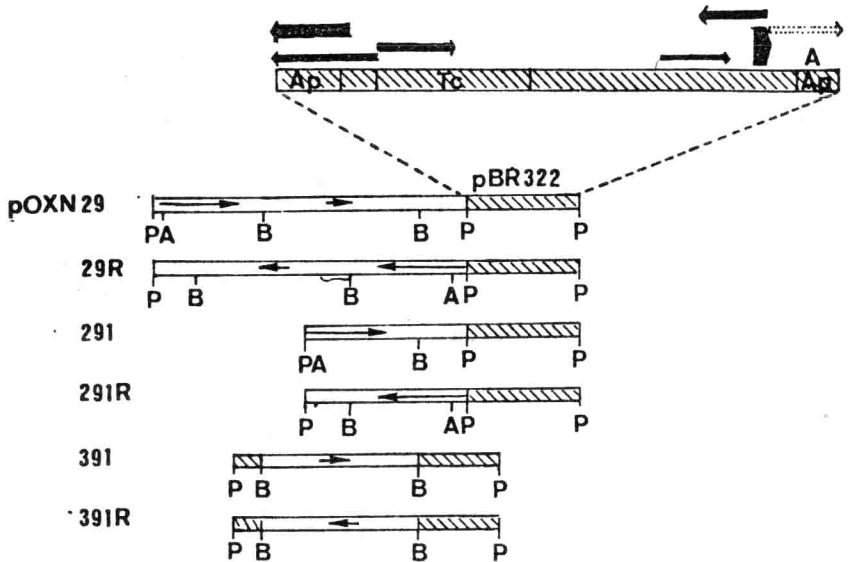


Fig.2 Relation between the structure of pOXN29 and its derivatives, and expression of β -xylosidase and xylanase in *E.coli*. The β -xylosidase productivity of pOXN29, 29R, 291, 291R, 391 and 391R are 0.92, 46.4, 1.0, 50.6, 0 and 0 respectively and that of xylanase of these plasmids are respectively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

11.4 and 6.3 kb by *Bgl*III 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 xylanase negative, indicating the locus of β -xylosidase within the 11.4 kb *Bgl*III fragment. This hybrid plasmid was named pOXN291. The 6.3 kb *Bgl*III fragment was subcloned into the *Bam*HI site 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
<u>E.coli</u> (pOXN29R) (osmotic shock)				
Xylanase	0	2	5	93
β -Xylosidase	-	4	4	92
β -Galactosidase	-	9	0	91
<u>E.coli</u> (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 deletion 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^+ ,.

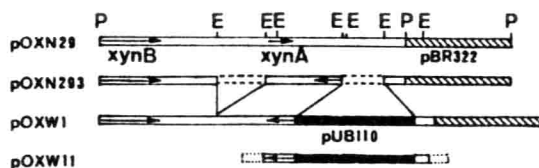


Fig 3. Structure of plasmids pOXN293, pOXW1, pOXW11 and pOXW12

pOXW1 was introduced into B.subtilis 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 deleted 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 M1111 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, xylanase 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 xylanase 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)	(m unit/mg)	
		Intra- cellular	Extra- cellular
<u>B. pumilus</u> IPO	16	0.39	600
<u>E. coli</u> C600	0	0	0
<u>E. coli</u> (pOXN29)	9.6	35	0
<u>E. coli</u> (pOXN293)	9.3	36	0
<u>E. coli</u> (pOXW1)	9.4	34	0
<u>B. subtilis</u> MI111	48	0.69	76
<u>B. subtilis</u> (pOXW11)	46	27	1,800
<u>B. subtilis</u> (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 deleted 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)			
	<u>B.pumilis</u>	<u>B.subtilis</u>	<u>E.coli</u>	<u>B. subtilis</u>
	IPO	MI111	(pOXN29)	(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


```

      20      40      60      75      90
GAATTCATTT CATCTTAGAG ATGACAGAAAT TAAAGGATG AAAAGGAGA GGATGACGA ATG AAT TTG AGA AAA TTA AGA CTG TTG TTT GTG ATG
      MET ASN LEU ARG LYS LEU ARG LEU LEU PHE VAL MET

      105      120      135      150      165      180
TGT ATT GGA CTG AGC ATT ATA CTG AGC GGT GTA CCA GTC CAT GCG AGA ACC ATT AEG AAT AAT GAA ATG GGT AAT CAG ACC GGG TAT
CYS ILE GLY LEU THR LEU ILE LEU THR ALA VAL PHE ALA HIS ALA ARG THR ILE THR ASN ASN GLU MET GLY ASN HIS SER GLY TYR

      195      210      225      240      255      270
GAT TAT GAA TTA TGG AAG GAT TAT GCA AAC AAT TCG ATG AFA FTY AAT AAC GCG GCG GCA TTT ACT GCA GCG TGG AAC AAT AIC GCA
ASP TYR GLU LEU TRP LYS ASP TYR GLY ASN THR SER MET THR LEU ASN ASN GLY GLY ALA PHE SER ALA GLY TRP ASN ASN ILE GLY

      285      300      315      330      345
AAT GGT TTA TTT AGA AAA GGG AAA AAG TTT GAT TCC ACT AGA AET CAC CAT CAG TTT GCG AAC ATA TCC ATC AAT TAC AAC GCA AGT
ASN ALA LEU PHE ARG LYS GLY LYS PHE ARG SER THR ARG THR HIS HIS GLN LEU GLY ASN ILE SER ILE ASN TYR ASN ILE ARG SER

      360      375      390      405      420      435
TIT AAC CCA AGC GGG AAT TCC TAT CTA TGT GTC TAT GCG TGG ACA TCT CCA TTA GCA GAA TAC TAT GAT GAT TCA TGG GCG
PHE ASN PRO SER GLY ASN SER TYR LEU CYS VAL TYR GLY TRP THR GLN SER PRO LEU ALA GLU TYR TYR ILE VAL ASP SER TRP GLY

      450      465      480      495      510      525
ACA TAT CGT CCA ACA GGA GCG TAT AAA GGA TCA TTT TAT GGT GAT GGA GCG ACA TAT GAC ATT TAT GAA ACA ACC CGT GTC AAT CAG
THR TYR ARG PRO THR GLY ALA TYR GLY SER PHE TYR ALA ASP GLY GLY THR TYR ASP ILE TYR GLU THR THR ARG VAL ASN GLN

      540      555      570      585      600      615
CCT TCC ATT ATE GGG ATC GCA ACC TTC AAG CAA TAT TGG AGT GTA CGT CAA AGC AAA CGT ACA AGC GGA AEG GTC TCC GTC AGC GCG
PRO SER ILE ILE GLY ILE ALA THR PHE LYS GLN TYR TRP SER VAL ARG GLN THR LYS ARG THR SER GLY THR VAL SER VAL SER ALA

      630      645      660      675      690      705
CAT TTT AGA AAA TGG GAA GGC TTA GGG ATG CCA ATG GCG AAA ATG TAT GAA AGC GCA TTT ACT GTA GAA GGC TAC CAA AGC AGC GGA
HIS PHE ARG LYS TRP GLU SER LEU SER LEU MET PHE MET GLY LYS MET TYR GLU THR ALA PHE TYR VAL GLU GLY TYR GLN SER SER GLY

      720      735      750      760      780      800
AGT GCA AAT GTG ATG ACC AAT CAG CTG TTT ATT GCG AAC TAAATA AGTCAAGAA AAGAGCGGG AGCAACATC CTGGCTTTT CTATCAAT T
SER ALA ASN VAL MET THR ASN GLN LEU PHE ILE GLY ASN

      820      840      860      880      900
TTTCACTT GCACTTGCC GGGAAAGAC GTTCCGAAA ACAAATGTCG ACGCCCGCC ATATCTGCA ACGCATGAG TGTGAGCCA TTCAACAAAT GTTTTT
TGC TTTTGTGTC TGCCCAATAT CTGTGCTGAC AACAAACCA GATAACACAT TTGTGTCGAC TGACAGCAIC AGCTTGCAIT CTCTCTGATG ATTCAACA

      920      940      960      980      1000
      1020      1040      1060
CGAACATGT CCCCATCTC AATCTGCTT TCTTTTGTCT CTGTGCAIT CATATGAGC

```

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 Ala²⁷ and Arg²⁸. 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 breed 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 dehydrogenase gene) promoter (Fig.5).