ENCYCLOPEDIA OF BIOPROCESS TECHNOLOGY: FERMENTATION, BIOCATALYSIS, AND BIOSEPARATION

VOLUME 3

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BIOPROCESS TECHNOLOGY: FERMENTATION, BIOCATALYSIS, AND BIOSEPARATION

VOLUME 3

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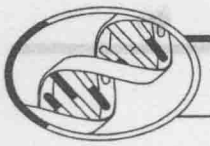

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VOLUME 3

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β-GALACTOSIDASE, ENZYMOLOGY

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KEY WORDS

Functional modification of bioactive compounds
Galactooligosaccharides
β-Galactosidase
Galactosyllactose
Glycosylhydrolase family
Lactase
Lactose hydrolysis
Microbial enzyme
Transgalactosylation
Reaction mechanism

OUTLINE

INTRODUCTION

β-Galactosidase (lactase, EC 3.2.1.23) catalyzes the hydrolysis and transgalactosylation reactions of β-D-galactopyranosides, such as lactose. The enzyme occurs in a wide variety of organisms including microorganisms, plants, and animals. The application of β-galactosidase in bioprocess technology has been achieved exclusively with microbial enzymes, which have long been used for the hydrolysis of lactose for increasing the digestibility of milk or for improving the functional properties of dairy products. During the past decade, another potential application of the enzyme has also been developed; the β-galactosidase-catalyzed transgalactosylation has proved to be useful for structural and functional modifications of food materials, medicines, and other biologically active compounds. In this article, we survey the enzymology and application in bioprocess technology of the microbial β-galactosidases with special emphasis on application of their transgalactosylation.

BRIEF SURVEY OF β-GALACTOSIDASE ENZYMOLOGY

Microbial Distribution and Purification

β-Galactosidase occurs in a variety of microorganisms including yeasts, molds, bacteria, actinomycetes, and archaebacteria (Table 1). β-Galactosidase is known as a marker enzyme for coliform bacteria, which are indicators of the fecal pollution of water.

Microbial β-galactosidases have been purified by a combination of several conventional techniques, such as salting-out fractionation, ion exchange, gel filtration, hydroxyapatite, and hydrophobic interaction chromatographies. The affinity chromatography of β-galactosidase uses *p*-aminophenyl- (or *p*-aminobenzyl-)β-D-thiogalactopyranoside agarose (1), which is commercially available and has been used for the purification of the *Escherichia coli lacZ* β-galactosidase, its recombinant derivatives, and the other β-galactosidases (2).

Molecular Properties, Substrates, and Inhibitors

The specificity and molecular properties of the microbial β-galactosidases differ significantly with the source of the enzyme. For example, the *Saccharopolyspora rectivirgula* β-galactosidase is highly specific for β-D-galactosides (3), whereas the *Sulfolobus solfataricus* enzyme shows broad substrate specificity and can efficiently hydrolyze β-glycosides other than β-galactosides as well (4). The catalytic competence and specificity of transgalactosylation also depend on the enzyme (see following). The reported native molecular weights and subunit structures of β-

Table 1. Examples of β-Galactosidase-Producing Microorganisms

Yeasts

Candida pseudotropicalis, Cryptococcus laurentii, Kluyveromyces (Saccharomyces) lactis, K. fragilis, K. marxianus, Lipomyces sp., *Torulopsis sphaerica, T. versalitis*

Molds

Altenaria altenata, Aspergillus awamori*, A. cellulosae*, A. foetidus*, A. nidulans, A. niger, A. oryzae, A. phoenicis*, A. terreus,* A. wentii*, Aspergillus* sp., *Chaetomium globosum*, C. cochlioides*, C. funicola*, C. thermophile* var. *coprophile*, Fusarium maniliforme, F. oxysporum* var. *lini, Geotricum candida*, Humicola grisea* var. *thermoidea*, H. lanuginosa*, Macrophomina phaseoli, Malbranchea pulchella* var. *sulfurea*, Mucor miehei*, M. pusillus*, Mucor* sp.*, *Mucor muccedo*, M. javanicus*, Neurospora crassa, Paecilomyces varioti, Penicillium* sp., *P. multicolor, P. canescens, P. citrinum, P. luteum*, P. chrysogenum*, P. frequentans*, P. cycropium*, P. toxidarium*, P. glaucum*, P. notatum*, P. roqueforti*, Phycomyces blakeleeanus, Rhizopus acidus*, R. niveus*, R. nigricans*, R. delemar*, R. javanicus*, R. formosciensis*, R. chinensis*, Scopulariopsis* sp., *Sclerotium tuliparum, Spicaria* sp., *Sporotrichum* sp.*, *S. thermophile*, Sterigmatomyces elviae, Thermomyces lanuginosus, Torula thermophila*, Trichoderma viride**

Basidiomycetes

*Corticium rolfsii, Culvularia inaequalis, Pycnoporus cinnabarinus, Sporobolomyces singulalis**

Bacteria

Gram-negative

Aeromonas cavie, A. formicans, Agrobacterium rediobacter, Bacteroides polypragmatus, Buttiauxella agrestis, Enterobacter (Aerobacter) cloacae, Escherichia coli, Fibrobacter succinogenes, Klebsiella pneumoniae, Rhizobium meliloti, R. trifolii, Shigella dysenteriae*, Thermotoga maritima, Thermus* sp., *Treponema phagedenis, Xanthomonas campestris, X. manihotis*

Gram-positive

Arthrobacter sp., *Bacillus acidocaldarius, B. circulans, B. coagulans, B. macerans, B. megaterium, B. subtilis, B. stearothermophilus,* alkalophilic *Bacillus, Bifidobacterium* sp., *B. bifidum*, B. longum, Clostridium acetobutylicum, Corynebacterium murisepticum, Lactobacillus delbruckii* subsp. *bulgaricus, L. bulgaricus, L. casei, L. helveticus, L. murinus, L. plantarum, L. sake, Lactococcus lactis, Leuconostoc citrovorum, L. lactis, Streptococcus salivarius* subsp. *thermophilus, S. thermophilus, S. (Diplococcus) pneumoniae, Thermoanaerobacter* sp., *Thermoanaerobacterium (Clostridium) thermosulfurigenes*

Actinomycetes

Actinomyces viscosus, Nocardia sp., *Saccharopolyspora rectivirgula, Streptomyces lividans*, S. venezuelae*, S. violaceus**

Archaebacteria

Caldariella acidophila, Sulfolobus solfataricus, Pyrococcus woesei, Haloferax alicantei

Note: Microorganisms listed include not only those whose β-galactosidase is characterized or sequenced but also those that have been only shown to produce β-galactosidase(s) based on the observed hydrolytic and transgalactosylation activities (indicated by an asterisk; May 1998).

galactosidases range, respectively, from 19,000 to 630,000 (the *Kluyveromyces lactis* enzyme [5]) and from monomer (e.g., the *S. rectivirgula* enzyme [3]) to heterooctamer (the *E. coli ebg* enzyme [6]). The bacterial β-galactosidases generally show optimum pHs at neutral regions whereas most mold enzymes show them at acidic regions, which in some cases reach pH 2 (7). Some β-galactosidases of the *lacZ* family require essential monovalent and divalent ions for their activities; the *S. rectivirgula* β-galactosidase is a multimetal enzyme that requires multiple divalent ions for its maximum thermostability and activity (8). However, some other β-galactosidases (e.g., the *Rhizobium meliloti* enzyme [9]) do not show such metal ion requirements. These observations strongly suggest the structural diversity of the microbial β-galactosidases.

Many chromogenic, fluorogenic, and luminogenic substrates that are specific for β-galactosidase have been designed (Table 2). D-Galactose, D-galactosylamines, β-D-thiogalactosides, and their analogs and derivatives serve as specific inhibitors of some microbial β-galactosidases (Table 3).

Structural Classification

Henrissat has compared all the available sequences of glycosyl hydrolases using hydrophobic cluster analysis and classified these enzymes into families (48). Many microbial β-galactosidases have thus far been cloned and sequenced and have been classified, on the basis of sequence similarities, into at least four categories according to Henrissat's classification (Table 4).

Glycosyl Hydrolase Family 2. β-Galactosidases belonging to this glycosyl hydrolase family, typified by the *E. coli lacZ* β-galactosidase, consist of a large subunit protein of approximately 1,000 amino acids and show very high sequence similarities to the animal β-glucuronidases, suggesting that the β-galactosidases of family 2 have a close evolutionary relationship with the β-glucuronidases (48). Many β-galactosidases of this family require monovalent and divalent metal ions for maximum activity (e.g., the *E. coli lacZ, Kluyveromyces lactis, Streptococcus thermophilus*, and *Saccharopolyspora rectivirgula* enzymes).

Table 2. Chromogenic, Fluorogenic, and Luminogenic Substrates for β-Galactosidase Gal, β-D-Galactopyranoside

A. Chromogenic Substrates

Substrate	Reported molar absorption coefficient of chromophore[a], M^{-1} cm^{-1}	Reference
2-Nitrophenyl-Gal (ONPG)	$\Delta\epsilon_{420}$ = 3000 (pH 7.0)	10
	$\Delta\epsilon_{410}$ = 2100 (pH 7.0)	11
	$\Delta\epsilon_{410}$ = 4500 (pH 8.6)	11
	$\Delta\epsilon_{410}$ = 4600 (pH 9.2)	11
4-Nitrophenyl-Gal (PNPG)	$\Delta\epsilon_{347,iso}$ = 290.4[b]	12
	$\Delta\epsilon_{405}$ = 13400 (pH 7.2)	3
	$\Delta\epsilon_{405}$ = 8900 (pH 7.0)	11
	$\Delta\epsilon_{405}$ = 18300 (pH 8.6)	11
3,4-Dinitrophenyl-Gal	$\Delta\epsilon_{400}$ = 15924 (pH 7.0[c])	12
2-Chloro-4-nitrophenyl-Gal		13
X-Gal[d]	Blue insoluble product	Suitable for histochemical identification
VBzTM-Gal[e]	Red product, $\Delta\epsilon_{520}$ = 55000	Suitable for histochemical identification; Refs. 14 and 15
VLM-Gal[f] and derivative	$\Delta\epsilon_{545}$ = 32500 (VLM-Gal)	15
VQM-Gal[g] and derivative	$\Delta\epsilon_{520}$ = 42500 (VQM-Gal)	15
6-Bromo-2-naphthyl-Gal	Product visualized by subsequent reactions	Suitable for activity staining in the gel; Refs. 16 and 17

B. Fluorogenic Substrates

Substrate	Excitation (nm)	Emission (nm)	Reference
FMG[h], FDG[i]	485	530	18
4-Hydroxy-4-methylcoumarin Gal	365	450	19
7-Hydroxycoumarine-4-acetate Gal	370	455	19

C. Substrates for Luminescent Determination

Substrate	Comments	Reference
D-Luciferin-*O*-Gal	Bioluminogenic	20
AMPGD[j]	Chemiluminogenic	21
Lumi-Gal 530™	Chemiluminogenic	22

[a]The value depends on assay conditions used. See reference for further details.
[b]Isosbestic point.
[c]Calculated from the reported equation: $\Delta\epsilon_{400} = (1.637 \pm 0.007) \times 10^4/(1 + [H^+]/10^{-5.45 \pm 0.02})$.
[d]5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside.
[e](2-(2-(4-(β-D-Galactopyranosyloxy)-3-methoxyphenyl)-vinyl)-3-methylbenzothiazoliumtoluene-4-sulfonate).
[f]4-[2-(4-β-D-galactopuyranosyloxy-3-methoxyphenyl)-vinyl]-1-methylquinoliniumiodide.
[g]2-[2-(4-β-D-galactopuyranosyloxy-3-methoxyphenyl)-vinyl]-1-methylquinoliniumiodide.
[h]Fluorescein mono-β-D-galactoside.
[i]Fluorescein di-β-D-galactoside.
[j](3-(4-Methoxyspiro[1,2-dioxetane-3,2′-tricyclo[3.2.1.1^{3,7}[decane]-4-yl)phenyl-β-D-galactopyranoside.

Glycosyl Hydrolase Family 1. The family 1 β-galactosidases include the enzyme of an archaeon, *Sulfolobus solfataricus* (48). This enzyme is essentially a β-glycosidase that can efficiently hydrolyze β-galactosides as well as the other β-glycosides (4). The *S. solfataricus* enzyme, consisting of 489 amino acid residues (49), differs in subunit size from the family 2 of enzymes and exhibits no sequence similarity to that family. Interestingly, the lactase/phlorizin hydrolase, a mammalian β-galactosidase playing a central role in lactose digestion in the small intestine, belongs to family 1 (48). Thus, it might be possible that family 2 β-galactosidases evolved to the animal β-glucuronidases whereas family 1 enzymes evolved to the digestive lactase enzymes functioning in the mammalian intestines. A comparison of stereo- and primary structures suggested that, along with β-glucosidases, cellulases, xylanases, and glycanases, the β-galactosidases belonging to families 1 and 2 form a superfamily, the 4/7 superfamily, which is characteristic of a common eightfold β/α architecture with two conserved, catalytically important glutamates near the C-terminal ends of β-strands four and seven (50).

The *lacG* β-Galactosidases. Strains of *Arthrobacter* sp. and *Bacillus stearothermophilus* produce β-galactosidases belonging to a recently proposed β-galactosidase family, the *lacG* family, which shows no sequence similarity to enzyme families 1 and 2 (51).

Glycosyl Hydrolase Family 35. *Xanthomonas manihotis*, which is a gram-negative phytopathogenic bacterium, *Arthrobacter* sp., *Bacillus circulans*, and *Aspergillus niger* produce β-galactosidases with strong sequence similarities to animal β-galactosidases belonging to family 35 of glycosyl hydrolases (52–54). Domain 1 of the *Xanthomonas*

Table 3. Specific β-Galactosidase Inhibitors

Inhibitor	Enzyme[a]	Type[b]	K_i and other properties[c]	Reference
D-Galactose	1	C	34 mM	23
	2	C	2.32 mM	3
	3	C	350 mM	24
	4	N	40 mM	25
D-Galactal	1	C	14 μM	26
D-Galactosamine	2	C	2.7 mM	3
2-Amino-D-galactopyranose[d]	1	C	1 mM	27
Galactobiose [β-D-Galp-(1,4)-D-Gal]	1	C	0.1 mM	28
β-D-Thiogalactosides	1	C	Isopropyl-, 0.085 mM	29
	2	C	Methyl-, 12 mM Isopropyl-, 15 mM	3
β-D-Galactopyranosyl trimethylammonium bromide[d]	1	C	1.4 mM	30
C-(β-D-Galactopyranosylmethyl) amine[d] and derivatives	1	C	7.8 μM	31
Furanoses[d]	1		L-Ribose, 0.03 mM D-Lyxose, 0.09 mM	23
D-Galactonolactones[d]	1		D-Galactonolactone, 0.25 mM	23
Amino sugars and amino alcohols[d]	1			27
Galactostatin and derivatives[d]	1	C	4 nM	32
Galactose-type imino sugar[d]	4		4 nM	33
1,5-Dideoxy-1,5-imino-D-galactitol[d]	1		13 μM	34
5-Amino-5-deoxy-D-galactopyranoside	1		45 nM	34
2′-Amino-2′-deoxymethyl β-lactoside	1			35
N-Bromoacetyl-β-D-galactopyranosylamine	1	IRR		36
β-D-Galactopyranosylmethyl-*p*-nitrophenyltriazene	4	A		37
(1/2,5,6)-2-(3-Azibutylthio)-5,6-epoxy-3-cyclohexen-1-*ol*	1	A	29 mM	38
Conduritol C *cis*-epoxide	1	A		39
2-Deoxy-2-fluoro-β-D-galactopyranosyl fluoride	1	A	K_i, 1.3 mM; k_i, 3.2 min^{-1}	40
	4	A	K_i, 5.4 mM; k_i, 2.5 min^{-1}	
	5	A	K_i, 1.3 mM; k_i, 0.8 min^{-1}	
2′,4′-Dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside	1	A	K_i, 0.71 mM; k_i, 2.4 min^{-1}	41
Diazomethyl β-D-galactopyranosyl ketone	4	A	K_i, 30 mM; k_i, 0.56 min^{-1}	42
2-Nitro-1-(4,5-dimethoxy-2-nitrophenyl) ethyl	1	IRR	Photoreversible thiol label	43
Phytic acid	1	U	3.46 mM	44
L-Histidine				45
Calystegin B1	6	C	1.6 μM	46
Isoflavones and their glycosides	6	C/N	2′,3′,4′,7-Tetrahydroxy-isoflavone, 26 μM	47
	6	C/N	Genistein 4′,7-di-*O*-α-L-rhamnoside, 4 μM	

[a]Sources of enzymes: 1, *Escherichia coli (lacZ)*; 2, *Saccharopolyspora rectivirgula*; 3, *Streptococcus thermophilus*; 4, *Aspergillus oryzae*; 5, *A. niger*; and 6, bovine liver. For the assay conditions, see References.
[b]Type of inhibition: C, competitive; N, noncompetitive; U, uncompetitive; IRR, irreversible; A, affinity labeling reagent.
[c]K_i, Inhibition constant; k_i, inactivation rate constant.
[d]Observed strong inhibitions by these compounds argue for the occurrence of a half-chair cationic transition state during catalysis.

manihotis β-galactosidase shows similarity to regions within catalytic domains of cellulases and xylanases belonging to family 10 of glycosyl hydrolases.

It should be mentioned that the strain of *Arthrobacter* sp. can produce β-galactosidases of different enzyme families, that is, families 1 and 35 as well as the *lacG* family (51,52,55). Cumulative knowledge of amino acid sequences and stereostructures of the microbial β-galactosidases will provide important information for further insights into the classification and evolutionary and structure–function relationships of the microbial β-galactosidases.

MOLECULAR PROPERTIES AND CURRENT STATUS IN BIOPROCESS TECHNOLOGY OF SOME MICROBIAL β-GALACTOSIDASES

Although many β-galactosidase-producing microorganisms are known (see Table 1), only a limited number of microorganisms have been selected as sources of the enzymes used in industry. Most of these are of fungal origin, such as *Kluyveromyces lactis, K. fragilis, Aspergillus niger*, and *A. oryzae*. These fungi were chosen mainly because they can inexpensively produce the β-galactosidase and are generally recognized as safe (GRAS) as food additives. Recent extensive studies led to discovering many other fungal and bacterial enzymes that are applicable in bioprocess technology. In this section, we describe the molecular properties and current status in bioprocess technology of some microbial β-galactosidases (Table 5).

Fungal Enzymes

The *Kluyveromyces lactis, K. fragilis, Aspergillus niger*, and *A. oryzae* β-galactosidases, the commercially available GRAS enzymes, are currently used in industry for manufacturing milk and dairy products (see following). The *Pae-*

Table 4. List of GenBank Accession Numbers of the Microbial β-Galactosidase Sequences (May 1998) with Possible Assignment to the Glycosyl Hydrolase Families

Microorganism (enzyme)	GenBank accession number	Possible assignment[a]
Aspergillus niger (*lacA*)	A00968, L06037 S37150	35
Actinobacillus pleuropneumoniae (*lacZ*)	U62625	2
Arthrobacter sp. (*lacZ*)	U12334	2
	U78028	35
	U17417	*lacG*
Bacillus circulans (*bgaA*)	L03424	*lacG*
(*bgaB*)	L03425	*lacG* or 35
(*bgaC*)	D88750	35
B. stearothermophilus	M13466	*lacG*
Clostridium acetobutylicum (*cbgA*)	M35107	2
C. perfringens (*pbg*)	D49537	
C. (Thermoanaerobacter) thermosulfurigenes (*lacZ*)	M57579	2
Enterobacter cloacae	D42077	2
Escherichia coli (*lacZ*)	V00296	2
(*ebg*)	X52031	2
Kluyveromyces lactis (*LAC4*)	M84410	2
Lactobacillus acidophilus (*lacL*)	AB004867	2
(*lacM*)	AB004868	2
L. delbrueckii subsp. *bulgaricus*	M23530	2
Lactococcus lactis (*lacZ*)	U60828	2
Leuconostoc lactis	M92281	2
Pyrococcus woesei	AF043283	1
Rhizobium meliloti (*lacZ*)	L20757	
Saccharopolyspora rectivirgula	D86429	2
Streptococcus thermophilus (*lacZ*)	M63636	2
Sulfolobus solfataricus	M34696, X15950, X15372	1
Thermotoga maritima	U08186	2
Thermus sp.	Z93773	*lacG*
Xanthomonas manihotis (*Bga*)	L35444	35

[a]For details, see text.

cilomyces varioti β-galactosidase is a thermostable extracellular enzyme with a pH optimum at the acidic region and is potentially applicable to the processing of acid whey. The *A. oryzae*, *Cryptococcus laurentii*, and *Sterigmatomyces elviae* enzymes exhibit high transgalactosylation activities and are used for the production of the "galactooligosaccharides," which are derived from lactose (see following).

Kluyveromyces lactis. Among the fungal enzymes listed in Table 5, biochemical and genetic studies have been the most intensive with the *K. lactis* enzyme. The *K. lactis* β-galactosidase is encoded by the *LAC4* gene, whose regulation involves the galactose/lactose induction and catabolite repression systems (64). The *K. lactis* enzyme consists of a subunit protein with an M_r of 117,618 (64) and is probably a dimer judging from its sedimentation coefficient under nondenaturing conditions (56), although higher aggregates are also known. The deduced amino acid sequence of the β-galactosidase is very similar to that of the *E. coli lacZ* enzyme; thus, the enzyme belongs to the family 2. However, Maxilact, a commercial preparation of the *K. lactis* enzyme, was shown to contain four enzyme forms of β-galactosidase, whose native molecular weights were estimated to be 19,000, 41,000, 550,000, and 630,000, respectively (5). The biochemical relevance of these observations to the reported subunit size (M_r, 117,618) and structure remains to be clarified.

Aspergillus niger. *A. niger* produces three forms of β-galactosidases with M_r of 124,000, 150,000, and 173,000, respectively, which can be separated by ion exchange, gel filtration, and hydrophobic interaction chromatographies (57). These isoforms share very similar isoelectric points, amino acid compositions, and kinetic parameters. The observed multiplicity seems to vary with the culture conditions and is probably related to differences in the carbohydrate content. The amino acid sequence of the *A. niger* β-galactosidase exhibits similarity to animal β-galactosidases belonging to the glycosyl hydrolase family 35 (65) (see Table 4).

Crypotococcus laurentii* and *Sterigmatomyces elviae. *C. laurentii* and *S. elviae* have been found to produce β-galactosidases that have high galactosyl transfer activities (2,62). The *C. laurentii* cells entrapped in the calcium alginate gels are currently used for industrial-scale production of 4′-galactosyllactose-containing "galactooligosaccharides" (see following).

The *C. laurentii* and *S. elviae* β-galactosidases share the following similar characteristics: *(1)* occurrence in the cell wall fractions of the yeast cells; *(2)* homodimer of a subunit protein with an M_r of 86,000–100,000; *(3)* optimum pH for hydrolysis reaction at pH 4.3–5.0; *(4)* high transgalactosylation activity that yields 4′-galactosyllactose as a main product from lactose; and *(5)* strong inhibition by Hg^{2+} but

Table 5. Molecular Properties of Some Microbial β-Galactosidases of Biotechnological Interest

Enzyme	Native M_r[a] (subunit structure)	Optimum pH[b]	Transfer activity[c]	Metal ion requirements	Localization[d]	Comments
			Fungal enzymes			
Kluyveromyces lactis	nr (probably dimeric)	$7.1^{lac,onpg}$	+	Mn^{2+}/Na^+	I	Subunit M_r, 117, 618; Refs. 56 and 57
K. fragilis	203,000 (nr)	6.8^{onpg}	+	Co^{2+}, Mg^{2+}, Mn^{2+}/K^+	I	Ref. 58
Aspergillus niger	124,000–173,000 (nr)	3.0^{lac}	+	nr	I	Three multiple forms; Ref. 57
A. oryzae	105,000 (homodimer)	4.5^{lac}	+ +	no	E	Refs. 59 and 60
Paecilomyces varioti	94,000 (nr)	3.5	+	no	E	Thermostable enzyme; Ref. 61
Sterigmatomyces elviae	170,000 (homodimer)	$4.5–5.0^{onpg}$	+ +	no	I (cell wall)	Thermostable enzyme; Ref. 2
Cryptococcus laurentii	200,000 (homodimer)	4.3^{onpg}	+ +	no	I (cell wall)	Ref. 62
			Bacterial enzymes			
Escherichia coli	465,000 (homotetramer)	7.0^{onpg}	+	Mg^{2+}, Mn^{2+}/Na^+	I	The *lacZ* enzyme
Bacillus circulans	160,000 (monomer)	6.1^{lac}	+ +	no	I	Isozyme 2; Ref. 63
Saccharopolyspora rectivirgula	145,000 (monomer)	7.1^{lac}	+ +	Mn^{2+}/Na^+	E	Thermostable enzyme; Ref. 3
Streptococcus thermophilus	282,000 (probably dimeric)	7.0	+ +	Mg^{2+}/K^+, Na^+	I	Ref. 24

Note: nr, not reported; no, no requirement.
[a]Molecular weight under nondenaturing conditions.
[b]Optimum pH for hydrolysis of lactose^{lac} or 2-nitrophenyl-β-D-galactopyranoside^{onpg}.
[c]+, Transgalactosylation activity has been reported; + +, very high transgalactosylation activity has been reported.
[d]I, intracellular, E, extracellular.

not by *p*-chloromercuribenzoic acid. However, the *S. elviae* enzyme is distinct from the *C. laurentii* enzyme in terms of thermostability; the *S. elviae* enzyme is stable up to 80 °C for 1 h although *S. elviae* is a mesophile; in contrast, the *C. laurentii* enzyme is inactivated after incubation at 58 °C for 10 min. The *S. elviae* enzyme exhibits sevenfold higher β-D-fucosidase activity than β-D-galactosidase activity (2).

Bacterial and Actinomycetous Enzymes

Escherichia coli. The *E. coli lacZ* β-galactosidase is the best characterized β-galactosidase; its three-dimensional structure was already known (66). The regulation mechanism of the transcription of the *lacZ* β-galactosidase gene was established as the operon model. The extensive structural and mechanistic studies of the *E. coli lacZ* enzyme have provided the basis for the detailed insights into β-galactosidase catalysis. However, the usefulness of this enzyme in bioprocess technology has been examined only on a laboratory scale; the *E.coli lacZ* enzyme stops short of making a contribution in the food industry, mainly because of the unacceptability of the bacterium in uses related to food. Rather, the *E. coli lacZ* β-galactosidase and its gene are essential tools in genetic engineering, immunochemical, and molecular biology studies.

The *E. coli lacZ* β-galactosidase is a tetrameric protein consisting of identical subunits of a 1,023-amino acid polypeptide chain, which folds into five sequential domains with an extended segment at the amino terminus (66). The enzyme has four active sites per tetramer, each of which is made up of elements from two different subunits. All the catalytically important amino acid residues are located in domain 3 of the subunit. The enzyme requires the essential divalent and monovalent cations for its maximum activity, but the role of these metal activators in the enzyme mechanism is not yet known in detail. The proposed mechanism of the hydrolysis and transgalactosylation reactions catalyzed by the *E. coli lacZ* β-galactosidase are reviewed in another section. The "α-complementation" phenomenon would be worth noting as a unique characteristic of this enzyme (67,68). A fragment of amino-terminal peptide, which consists of approximately 50 amino acids encoded by a plasmid, is capable of intraallelic (α-) complementation with a defective form of the β-galactosidase encoded by the host. The α-complementation has been used for designing plasmid vectors that permit histochemical identification of recombinant clones in genetic engineering studies.

The *E. coli* cells produce a second type of β-galactosidase, the *ebg* β-galactosidase ("*ebg*" denotes "evolved β-galactosidase"), which was found as a gene product that confers a lactose-positive phenotype to *lacZ*$^-$ mutants. The enzyme is encoded by two distinct structural genes of the *ebg* operon, *ebgA* and *ebgC* genes that encode polypeptide chains of M_r 110,000 and 20,000, respectively (6). The sequence of the *ebgA* gene shows 50% nucleotide identity with that of the *lacZ* β-galactosidase gene, indicating that

the *ebg* β-galactosidase has a strong evolutionary relationship with the *lacZ* β-galactosidase (69). The active form of the *ebg* β-galactosidase is an $\alpha_4\beta_4$ heterooctamer, which is a 1:1 complex of the *ebgA* and *ebgC* gene products (6). Because the wild-type *ebg* β-galactosidase is too catalytically feeble to allow the *E. coli* cells to grow on lactose, spontaneous mutations must occur in the *ebg* β-galactosidase genes before growth can be sustained; when the *lacZ*$^-$ mutants are placed under selection pressure with lactose as a sole carbon source, strains producing mutant *ebg* enzyme with enhanced catalytic competence are selected. Thus, although the *ebg* β-galactosidase has made no significant contribution to bioprocess technology to date, the *ebg* system has been extensively studied as an interesting model for the biology of acquisitive evolution and the chemistry of the evolution of catalytic function.

Bacillus circulans. The commercial preparation of *B. circulans* β-galactosidase contains two isozymes, isozymes 1 and 2, which are reported to be monomeric with M_r of 240,000 and 160,000, respectively (63). The enzyme preparation could be applicable to enzymatic hydrolysis of lactose in skim milk (70). The isozymes are different from each other in terms of substrate specificity and the ability to catalyze transgalactosylation. Isozyme 2 has an extremely high transgalactosylation activity; the reaction of the enzyme with 4.56% (w/v) lactose yields the transgalactosylation products (galactooligosaccharides) consisting of di-, tri-, tetra-, and pentasaccharides, and the amount of the oligosaccharides reaches the maximum, which is 41% (w/w) of the total sugar in the reaction mixture. In contrast, isozyme 1 produces oligosaccharides whose amount is only 6% (w/w) of the total sugar at maximum under the same conditions. Thus, isozyme 2 of *B. circulans* β-galactosidase is used for the production of "galactooligosaccharides" as well as for the functional modification of food materials, medicines, and other bioactive compounds by transgalactosylation. Two β-galactosidase-encoding genes, *bgaA* and *bgaB*, have been identified in *B. circulans* (see Table 4) and are potential candidates that code for these isozymes. Relevance of these genes to the isozymes, however, still remains to be established. Recently, *B. circulans* has also been found to have a third β-galactosidase, *bgaC*, which is an *exo*-β-1,3-galactosidase belonging to glycosyl hydrolase family 35 (53). The commercial preparation of *B. circulans* β-galactosidase does not contain the activity of this third β-galactosidase (53). The recombinant *bgaC* β-galactosidase also showed transgalactosylation activity, which is useful for the synthesis of β-1,3-linked galactosyl oligosaccharides (71).

Streptococcus thermophilus. *S. thermophilus* is one of the dairy lactic streptococci that are used as starter cultures for a variety of industrial dairy fermentations such as those during the yogurt-making processes. The primary function of these dairy lactic streptococci is the rapid conversion of lactose into lactic acid, an essential process in dairy fermentation (72). Two distinct biochemical pathways involved in lactose utilization by the lactic streptococci have been established. One of these pathways uses the lactose permease system for the transport of unmodified lactose, which is then hydrolyzed by an intracellular β-galactosidase. The other pathway is mediated by the phosphoenolpyruvate-dependent phosphotransferase system, in which lactose is phosphorylated during translocation and the resultant lactose-6-phosphate is hydrolyzed by phospho-β-galactosidase (EC 3.2.1.85). In *S. thermophilus* cells, the lactose permease system plays a central role in lactose transport across the bacterial cell membrane, and the β-galactosidase functions as a key enzyme in the metabolism of the internalized lactose in dairy lactic fermentation (72). The *S. thermophilus* β-galactosidase is promising for industrial hydrolysis of milk lactose, judging from its thermostability and established safety as a food additive. Also, the enzyme is used as a catalyst for the industrial production of "galactooligosaccharides" from lactose because of its high transgalactosylation activity.

The *S. thermophilus* β-galactosidase consists of a subunit protein of M_r 116,860 (73). However, its quaternary structure is not yet firmly established. The enzyme exists in three distinct forms, whose apparent native molecular weights are 204,000, 186,000, and 282,000, and one of which is converted to another in a time-, temperature-, and concentration-dependent manner (24). The native molecular weight of 500,000–600,000 has also been reported (72). Structural analysis of the *S. thermophilus* β-galactosidase genes showed that the β-galactosidase is a family 2 enzyme with 48%, 35%, and 32.5% amino acid sequence identity to the *Lactobacillus bulgaricus, Escherichia coli,* and *Klebsiella pneumoniae* enzymes, respectively. The enzyme requires monovalent and divalent metal ions for its maximum activity, and the specificity of the metal activation varies with the substrate (24). The enzyme shows high transgalactosylation activity to produce a variety of disaccharides as main products from lactose (74), in contrast to the *Aaspergillus oryzae* β-galactosidase, for example, producing tri-, tetra-, and pentasaccharides as main transfer products from lactose.

Saccharopolyspora rectivirgula. A strain of *S. rectivirgula*, a thermophilic actinomycete, produces an extracellular thermostable β-galactosidase with high transgalactosylation activity. The *S. rectivirgula* β-galactosidase is a monomeric enzyme with a molecular weight of 145,000 and $S^\circ_{20,w}$ of 7.1*S* (3). When the enzyme reacts with 1.75 M lactose at 70 °C and pH 7.0 for 22 h, it yields oligosaccharides in the maximum yield (other than lactose) of 41% (w/w). The enzyme is stable at pH 7.2 up to 60 °C (for 4 h in the presence of 10 μM $MnCl_2$) or 70 °C (for 22 h in the presence of 1.75 M lactose and 10 μM $MnCl_2$). Thus the enzyme is applicable to an immobilized enzyme system at high temperatures (>60 °C) for efficient production of the oligosaccharides from lactose. This monomeric enzyme has eight specific binding sites for divalent metal ions. These sites are classified as follows: a very tight (class I) site for Ca^{2+}, three tight (class II) sites consisting of two Ca^{2+}-specific sites (class II_{Ca}) and one Mn^{2+}-specific site (class II_{Mn}; K_d for Mn^{2+}, 2.0 nM), and four loose (class III) sites for Mn^{2+} (K_d, 1.2 μM) and Mg^{2+} (K_d, 2 μM). The class II Mn^{2+} is catalytically important in maintaining the native structure essential for activity. Occupation of class III sites by Mg^{2+} or Mn^{2+} is of physiological importance to attain suf-

ficient thermostability by which this extracellular β-galactosidase remains active for a prolonged time at elevated temperatures, as was observed during the growth of *S. rectivirgula* (8).

MECHANISM OF THE *ESCHERICHIA COLI lacZ* β-GALACTOSIDASE-CATALYZED REACTIONS

The mechanism of the hydrolysis and transgalactosylation reactions has been the most intensively studied with the *Escherichia coli lacZ* β-galactosidase. Figure 1 shows the proposed kinetic mechanism of the *E. coli lacZ* β-galactosidase (75). In this mechanism, hydrolysis and transgalactosylation occur at the same catalytic site and share a common galactosyl enzyme intermediate. Extensive kinetic studies have also suggested the presence of two distinct subsites near the active site of the *lacZ* enzyme, the "galactose site" and "glucose site," that respectively bind galactose and glucose portions of the lactose molecule (76,77). The glucose site shows affinities to a variety of nucleophilic compounds, such as sugars, alcohols, and thiols. The presence of such a glucose site is important in the kinetic behaviors of hydrolysis and transgalactosylation of lactose.

Kinetic Mechanism of Reactions with ONPG

The kinetic mechanism of the *Escerichia coli lacZ* β-galactosidase-catalyzed reactions can be most simply shown by the reaction with 2-nitrophenyl β-D-galactopyranoside (ONPG), which is a synthetic substrate whose aglycone serves as a good leaving group (75). The ONPG (termed S in Fig. 1) binds to the enzyme to form a Michaelis complex ES (step a). The release of the aglycone, 2-nitrophenolate, occurs very rapidly (k_2 = 2,100 s^{-1} at 25 °C and pH 7.0) to yield a galactosyl enzyme intermediate (step b), which partitions into subsequent hydrolysis (c) and transgalactosylation (g, h). For the hydrolysis reaction, the water molecule reacts with the galactosyl enzyme intermediate with k_3 of 1,200 s^{-1}, yielding galactose and free enzyme (degalactosylation; step c). For the transgalactosylation, a galactosyl acceptor (termed Nu) present in the reaction mixture binds to a glucose site of the enzyme with an affinity of K_i'' to form a ternary complex (step g), followed by the formation of the galactosyl adduct (Gal-Nu; step h). In the transgalactosylation reaction, the apparent rate of the 2-nitrophenol release is increased, decreased, or unchanged with increasing acceptor concentrations, depending on the nature of the acceptor and the relative magnitude of k_2, k_3, and k_6. These observations have been kinetically analyzed to estimate the K_i'' and k_6 values of a variety of acceptors, which are the measures of how well they bind at the glucose site and how readily they react to form a galactosyl adduct, respectively. Mathematical considerations of such analyses are described by Deschavanne et al. (76) and Huber et al. (77).

Kinetic Mechanism of Reactions with Lactose

The reaction with lactose, a natural substrate, proceeds with a mechanism that is somewhat different from that of the ONPG reaction. Steps d, e, and f, which are shown with shadowing in Figure 1, must be considered instead of step b, because glucose, a product of lactose hydrolysis, has an affinity (K_i') to the glucose site, in contrast to 2-nitrophenolate, which has no affinity to the enzyme (75); released glucose can reversibly bind to form a ternary complex (step e), and then reacts with galactose to yield allolactose. During the lactose hydrolysis, allolactose is only transiently formed because it is also a substrate of the enzyme. The reported rate constants (at 25 °C, pH 7.0) are k_4 = 60 s^{-1}, k_5 = 380 s^{-1}, and k_3 = 1,200 s^{-1} (75). When high concentrations of lactose are reacted, lactose also serves as a galactosyl acceptor (Nu), and the transfer product is galactosyllactose. The galactosyllactose can, in turn, serve as a galactosyl acceptor to produce tetrasaccharides. In this manner, a mixture of oligosaccharides consisting of di-, tri-, and higher saccharides is produced from lactose.

Role of Active Site Residues in Catalysis

Extensive chemical modification, affinity labeling, and site-directed mutagenesis studies have been carried out to find functional amino acid residues at the active site of the *Escherichia coli lacZ* β-galactosidase; these have shown that Glu461, Glu537, and Tyr503 are important for en-

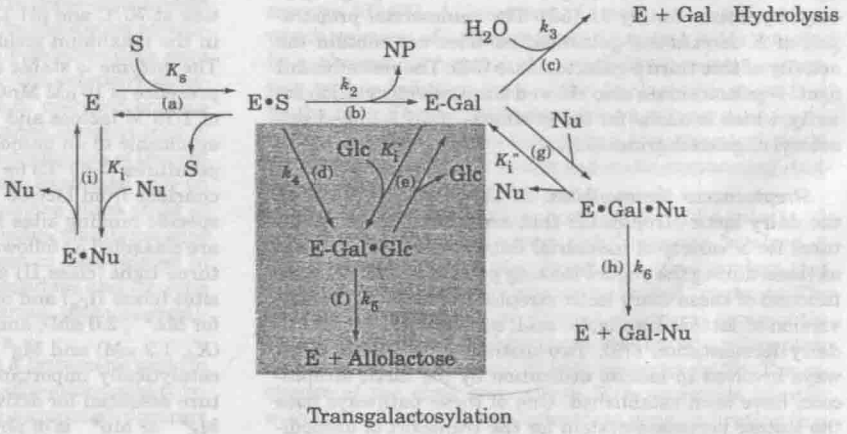

Figure 1. Proposed kinetic mechanism of hydrolysis and transgalactosylation reactions catalyzed by the *E. coli lacZ* β-galactosidase. *E*, enzyme; *S*, substrate; *NP*, 2-nitrophenol; *Gal*, galactose; *Glc*, glucose; *Nu*, galactosyl acceptor. Shadowed portion (steps *d*, *e*, *f*) indicates the pathway that must be considered, instead of step *b*, for the mechanism of the reaction with lactose. This mechanism also shows that the Nu also binds to free enzyme to form a deadend complex whose dissociation constant is *Ki* (step *i*).

zyme activity. All these catalytically important residues are located in the third domain of the enzyme subunit and, indeed, are shown to be located at the active site by X-ray crystallographic studies (66). However, some argument still exists on the details of the role(s) of each active site residue. Here we briefly describe the currently proposed roles of these amino acid residues in β-galactosidase catalysis (Fig. 2).

The glycosidic oxygen atom of the enzyme-bound substrate undergoes protonation, which facilitates aglycon departure. Intensive kinetic studies with site-directed mutants (17,78) suggest that the Glu461 serves as a Brønsted general acid–base catalyst at this step, although some evidence also argues for the role of Tyr503 being the general acid–base catalyst (79,80). The elimination of the aglycon probably produces an oxocarbonium ion intermediate that is planar at its anomeric end. This reaction is assumed because the planar and positively charged analogs of galactose serve as strong inhibitors of the *E. coli lacZ* β-galactosidase (75) (see Table 3). Affinity-labeling studies provided evidence suggesting that Glu537 is a nucleophile that attacks the anomeric carbon of the cationic intermediate (41), yielding a galactosyl enzyme in which the galactose moiety is covalently bound to the enzyme through an ester linkage. The water (or acceptor) then reacts with the covalent intermediate to produce galactose (or galactosyl adduct) and free enzyme. Although the enzyme requires monovalent and divalent ions for its activity, the role of the metal activators is not yet known. The bound Mg^{2+} (1 atom/active site; K_d, 0.65 μM [25]) has been suggested to act as an electrophilic catalyst that accelerates aglycone departure (12), whereas there is alternative evidence suggesting that it is only important in maintaining the proper active site structure (30,75). The Glu416, His418, and Glu461 are ligands to the bound Mg^{2+} ion (66).

APPLICATIONS OF β-GALACTOSIDASE-CATALYZED REACTIONS IN BIOPROCESS TECHNOLOGY

Hydrolysis

Cow milk generally contains 4.5–5.0% (w/v) lactose, whose low digestibility, low solubility, and low sweetness pose the following problems, the so-called lactose problems, in the milk and dairy industries (81).

1. Lactose intolerance is widespread in the world's adult population, with 70% being unable to metabolize large quantities of lactose in milk because of lack of intestinal lactase.
2. The high lactose content in nonfermented milk products, such as ice cream and condensed milk, often causes lactose crystallization during preservation. The crystals must not be greater than 10 μm in length, or they would make the products sandy.
3. The whey and whey permeate, which are the lactose-containing by-products from cheese manufacturing, have posed a serious problem as environmental pollutants, although these are potentially good carbohydrate sources for foods and animal feeds (82).

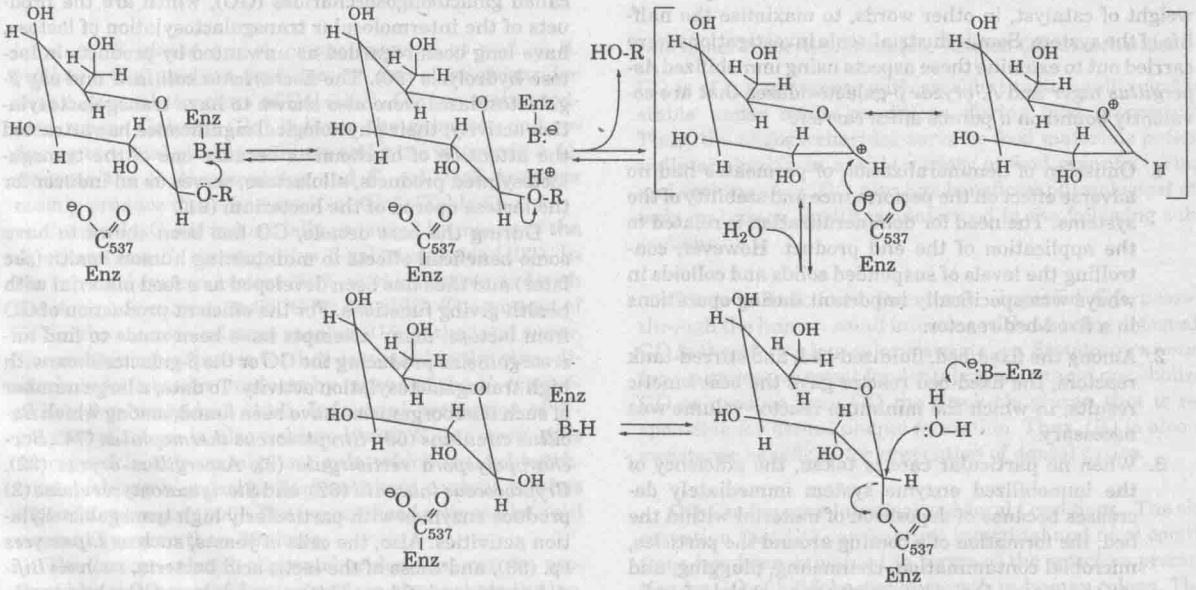

Figure 2. Catalytic mechanism of the *E. coli lacZ* β-galactosidase. Glu461 and Tyr503 are the candidates for the Brønsted general acid/base catalyst (Enz-BH/Enz-B:). A carboxyl group shown with "537" is the Glu537 that is suggested to act as a nucleophile during catalysis. The possible resonance of the planar cationic intermediate is shown with *square brackets*.

All these problems can be circumvented by decomposing lactose in the products by the action of β-galactosidase (83,84). For the purpose of alleviating lactose maldigestion, low-lactose milk is currently produced in industry by the continuous or batchwise treatment of milk with fungal β-galactosidases. Fungal β-galactosidase tablets have also been devised for this purpose and are commercially available (85,86). Enzymatic lactose hydrolysis can be considered to be a unit operation that can be integrated into the transformation of a wide range of whey and whey permeates (87). Hydrolysis of lactose produces a sugar mixture, glucose plus galactose, that is four to five times sweeter and three to four times more soluble than the parent disaccharide. The sugar mixture can serve as a condensed sugar syrup that is used as a sweetener or intermediate food product for manufacturing bakery and confectionary products, ice cream, and other specialities, permitting the efficient utilization of wheys and whey permeates (87).

Application of Immobilized Enzyme Technology in Industrial Lactose Hydrolysis

It is important to achieve industrial lactose hydrolysis with minimum cost. Immobilization of β-galactosidases is a means to reduce the cost of industrial lactose hydrolysis. Methods of immobilization and the properties of the immobilized β-galactosidases have been summarized (84). Baret (87) pointed out the following practical aspects in attaining the best performance of the immobilized enzyme system for the processing of whey and its permeates: (*1*) the nature of the substrate wheys and permeates, (*2*) the design of the reactor system, and (*3*) the operating strategy. The key objective of these considerations is to maximize the amount of hydrolyzed lactose per unit weight of catalyst, in other words, to maximize the half-life of the system. Semiindustrial-scale investigations were carried out to examine these aspects using immobilized *Aspergillus niger* and *A. oryzae* β-galactosidases that are covalently bound on a porous silica carrier:

1. Omission of demineralization of permeates had no adverse effect on the performance and stability of the systems. The need for demineralization is related to the application of the end product. However, controlling the levels of suspended solids and colloids in wheys was specifically important during operations in a fixed-bed reactor.
2. Among the fixed-bed, fluidized-bed, and stirred-tank reactors, the fixed-bed reactor gave the best kinetic results, in which the minimum reactor volume was necessary.
3. When no particular care is taken, the efficiency of the immobilized enzyme system immediately decreases because of deposition of material within the bed, the formation of a coating around the particles, microbial contamination, channeling, plugging, and other reasons. Operating strategy must therefore be considered to minimize these problems. The strategy required includes the removal of solids or colloidal substances in substrates, sanitation of substrates, introduction of the cycling of cleaning/sanitation procedures of the reactor during the operation, and programming the operating temperatures. These strategies greatly enhanced the activity and stability of the reactors.

It should be emphasized that the prevention against microbial contamination during operation is of specific importance for the processing of wheys and their permeates, which are nutritionally rich and excellent growth media for microorganisms. Methods for sanitizing the substrates, reactor, and other possible sources of microbial contamination were evaluated. Dilute acetic acid, which has been commonly used in the laboratory for disinfection of the immobilized enzyme matrices, served as only a poor sanitizer under industrial conditions. The substituted diethylenetriamines were effective for the disinfection of the immobilized enzyme matrices without causing loss of enzyme activity and have been used in large-scale operations.

The technology obtained from these large-scale examinations was transferred to fully industrial-scale operations; a plant processing 20,000 L/day of sweet whey has thus been operated, in which the hydrolysis reactor can process 1,000 L/h of nondemineralized acidified sweet whey using 40 kg of the immobilized β-galactosidase matrices (87). The processed products have been used as ingredients in the food industries.

Production of Lactose-Related Oligosaccharides by Transgalactosylation

It was shown in the 1950s that oligosaccharides are formed during the hydrolysis of lactose in milk with the fungal β-galactosidases (88,89). These oligosaccharides, the so-called galactooligosaccharides (GO), which are the products of the intermolecular transgalactosylation of lactose, have long been regarded as unwanted by-products in lactose hydrolysis (90). The *Escherichia coli lacZ* and *ebg* β-galactosidases were also shown to have transgalactosylation activity; their physiological significance has attracted the attention of biochemists because one of the transgalactosylated products, allolactose, serves as an inducer for the lactose operon of the bacterium (91).

During the past decade, GO has been shown to have some beneficial effects in maintaining human health (see later) and thus has been developed as a food material with health-giving functions. For the efficient production of GO from lactose, many attempts have been made to find microorganisms producing the GO or the β-galactosidase with high transgalactosylation activity. To date, a large number of such microorganisms have been found, among which *Bacillus circulans* (63), *Streptococcus thermophilus* (74), *Saccharopolyspora rectivirgula* (3), *Aspergillus oryzae* (92), *Cryptococcus laurentii* (62), and *Sterigmatomyces elviae* (2) produce enzymes with particularly high transgalactosylation activities. Also, the cells of yeasts, such as *Lipomyces* sp. (93), and those of the lactic acid bacteria, such as *Bifidobacterium bifidum* (94), can produce GO abundantly from lactose. Table 6 shows the identified structures of GO obtained with several enzymes and microbial cells. The structures of GO depend on the enzymes or microorganisms used as catalysts. The *B. circulans, S. rectivirgula, C.*

Table 6. Structures of Oligosaccharides Produced from Lactose by the Transgalactosylation Catalyzed by Microbial β-Galactosidases and Cells

Structure	Enzyme									Microorganism		
	1	2	3	4	5	6	7	8	9	10	11	12
β-D-Galp-(1→6)-D-Glc (allolactose))	●	●			●	●	●		●	●		●
β-D-Galp-(1→3)-D-Glc						●	●		●	●		●
β-D-Galp-(1→2)-D-Glc						●	●		●			
β-D-Galp-(1→6)-D-Gal		●			●	●	●			●		●
β-D-Galp-(1→3)-D-Gal						●	●					
β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glc (6′-galactosyllactose)								●		●		
β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glc (3′-galactosyllactose)								●			●	●
β-D-Galp-(1→4)-β-D-Galp-(1→4)-D-Glc (4′-galactosyllactose)			●	●					●		●	
β-D-Galp-(1→6)[β-D-Galp-(1→4)]-D-Glc					●			●	●			●
β-D-Galp-(1→2)[β-D-Galp-(1→4)]-D-Glc									●			
β-D-Galp-(1→2)[β-D-Galp-(1→6)]-D-Glc									●			●
β-D-Galp-(1→3)[β-D-Galp-(1→6)]-D-Glc									●			
β-D-Galp-(1→4)-β-D-Galp-(1→6)-D-Glc									●			
β-D-Galp-(1→6)-β-D-Galp-(1→6)-D-Glc					●							
β-D-Galp-(1→4)-β-D-Galp-(1→3)-D-Glc									●			
β-D-Galp-(1→4)-β-D-Galp-(1→2)-D-Glc									●			
β-D-Galp-(1→6)-β-D-Galp-(1→6)-D-Gal					●							
β-D-Galp(1→6)-β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glc					●			●				
β-D-Galp-(1→3)-β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glc								●				
β-D-Galp-(1→6)-β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glc								●				
β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glc												●
β-D-Galp-(1→6)-β-D-Galp-(1→6)-β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glc								●				
β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glc												●
β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glc												●
β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glc												●

Note: Closed circles indicate that the corresponding sugar has been identified in the transfer products. Enzymes are 1, *Escherichia coli* (Ref. 95); 2, *Streptococcus thermophilus* (Ref. 74); 3, *Sterigmatomyces elviae* (Ref. 2); 4, *Cryptococcus laurentii* (Ref. 62); 5, *Kluyveromyces lactis* (Ref. 96); 6, *K. fragilis* (Refs. 97,98); 7, *Aspergillus niger* (Ref. 98); 8, *A. oryzae* (Ref. 92); 9, *Bacillus circulans* (Ref. 99). Microorganisms are 10, *Penicillium chrysogenum* (Ref. 100); 11, *Chaetomium globosum* (Ref. 101); 12, *Bifidobacterium bifidum* (Ref. 94).

laurentii, *S. elviae*, and *A. oryzae* enzymes as well as *B. bifidum* cells produce from lactose the oligosaccharides with a general structure of Gal-$(Gal)_n$-Glc* as main products, where Gal and Glc* indicate the galactose and reducing terminal glucose residues and n the integer ($n \geq 1$), whereas the *S. thermophilus* and *E. coli lacZ* enzymes mainly produce disaccharides (Gal-Glc*) (Table 6).

Currently, GO is industrially produced by means of the *A. oryzae* and *S. thermophilus* β-galactosidases (102). In the reported system, lactose (80%, w/v) is first reacted with the *A. oryzae* enzyme at 65 °C for 4 h (103). The content of GO in the reaction mixture reaches 31% of the total sugar. The remaining lactose is further converted to the other disaccharidal transfer products by means of the *S. thermophilus* β-galactosidase (104). Industrial production of 4′-galactosyllactose is also achieved with *C. laurentii* cells entrapped in calcium alginate gels, which is used batchwise, yielding 4′-galactosyllactose whose content reaches 35% of total sugar (105). The immobilized cells can be used repeatedly, more than 30 times.

The lactose-related oligosaccharides that can be produced by the action of β-galactosidase also include the lactitol oligosaccharides, which are produced by the *A. oryzae* β-galactosidase-catalyzed transgalactosylation to lactitol [β-galactopyranosyl-(1→4)-D-glucitol], a reduced form of lactose (106).

Functional Properties of Lactose-Related Oligosaccharides

GO is of low sweetness with excellent taste quality and is stable under acidic conditions during food processing. Thus, the oligosaccharides serve as food materials potentially applicable to a wide variety of food manufacturing and cooking uses. GO also has beneficial physiological effects on human health, as described in the following subsections.

GO Is a Functional Sweetener. Ingested GO passes through the human small intestine without being digested; GO is therefore a low-calorie sweetener. *Streptococcus mutans*, a causative agent for dental caries, cannot metabolize GO or produce from GO the insoluble glucan that is responsible for dental plaque formation. Thus, GO is also a sweetener beneficial for prevention of dental caries.

GO Can Improve Intestinal and Fecal Conditions. The observation that GO can improve intestinal and fecal conditions has been explained in terms of the selective promotion by GO of bifidobacterial growth in human colons. The most dominant indigenous bacteria in the neonatal gut are the bifidobacteria, whose predominance in the gut has been suggested to cause beneficial effects in maintaining human health; these bacteria, as well as their metabolites,