MICROBIAL AMYLOLYTIC ENZYMES

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I. INTRODUCTION

There are in nature two widely distributed carbon sources, starch and cellulose, which maintain most forms of life. These polymers are composed of glucose units which have different anomeric conformations so that different enzymatic systems are needed to degrade them. Enzymes which are capable of hydrolyzing the α-glucosidic linkages of starch are called amylolytic enzymes, or α-glucanases, and the substrates are designated α-glucans.

Enzymes capable of hydrolyzing starch are produced by animals, plants, and microbes. α-Glucanase sources are numerous, and the properties of these enzymes differ widely. Organisms usually have several enzymes related to starch hydrolysis. Microbes are widely used for production and study of glucanases and structure of starchy materials.

In this review some features of amylolytic enzymes synthesized by microbes are considered. Several reviews describing starch-degrading enzymes have appeared,[1-15] but the molecular biology of these enzymes and respective genes has not been described in detail. Therefore, in this review we emphasize for enzymes having different action patterns on starch their physicochemical properties, occurrence, genetics, and results obtained from cloning of the genes. A consideration of α-amylase occupies the main part of this review because it is the best known amylolytic enzyme; indeed, most of the genetic and molecular biological studies on α-glucanases have been performed with α-amylase.

II. THE SUBSTRATE AND THE ENZYMES

Starch, which is produced mainly in higher plants, is composed of two components, amylose and amylopectin. Related α-glucan of animal origin is called glycogen. Amylose is a mainly linear polysaccharide which is formed by α-1,4-linked α-D-glucose residues and some α-1,6-branching points (see Figure 1). Amylopectin has a highly branched tree-like structure, and glycogen is even more branched than amylopectin. The proportion of branches is an important property of the substrate because enzymes hydrolyze different substrates with differing specificities. The relative content of amylose and amylopectin varies with the source of starch. Average chain length of amylose is about 1000 glucose units. The chain profile of amylopectins usually has a bimodal distribution with longer and shorter chains having average lengths of 40 to 60 and 11 to 25 D-glucosyl residues, respectively.[16-20] Amylolytic enzymes have been valuable tools in elucidating the structure of starch and glycogen.[21,22]

Several amylolytic enzymes hydrolyze starch or its degradation products. The actions of these enzymes can be divided in two categories. Endoamylases split linkages in random fashion in the interior of the starch molecule. Exoenzymes hydrolyze from the nonreducing

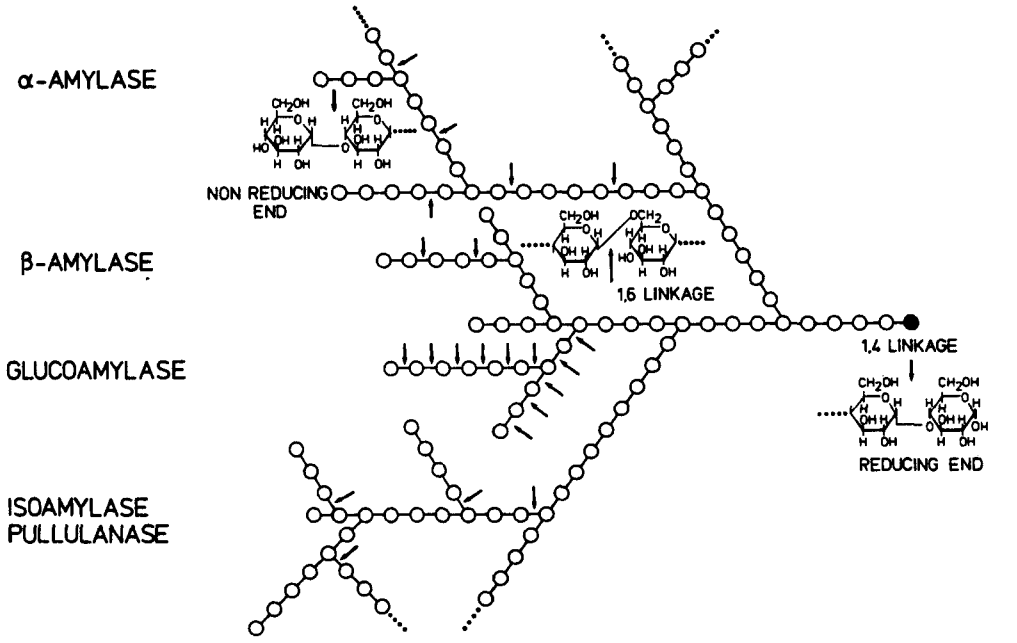

FIGURE 1. Schematic structure of amylopectin and action pattern of some amylolytic enzymes. The circles represent glucose units, and the arrows represent sites which the amylolytic enzymes can hydrolyze.

end (see Figure 1), successively resulting in short endproducts. Another division can be made according to which linkages the enzymes are capable of degrading; for example, debranching enzymes can hydrolyze 1,6-linkages. There exist more amylolytic enzymes than will be dealt with here, but they have not been well characterized. The enzymes described here are those having the best-known starch-degrading activities of microbial origin.

α-Amylase is an endoenzyme which hydrolyzes internal α-1,4-bonds and can bypass 1,6-linkages. β-Amylase is an exoenzyme which liberates maltose by hydrolyzing 1,4-linkages from the nonreducing end. Because it can not bypass 1,6-linkages, there always remain some β-limit dextrins after β-amylolysis. Glucoamylase produces glucose and can degrade both 1,4- and 1,6-linkages. Some other exoacting enzymes producing short oligosaccharides have also been found. Isoamylase and pullulanase are debranching enzymes which hydrolyze 1,6-linkages. They differ in ability to hydrolyze pullulan, a polysaccharide composed of 1,6-glycosidically linked maltotriose units. Only pullulanase can degrade this α-glucan. α-Glucosidases hydrolyze both 1,4- and 1,6-linkages, but usually only from short chain oligosaccharides formed by the action of other amylolytic enzymes. There is one additional class of amylolytic enzymes which deserves interest, specifically, the cyclodextrin-forming enzymes, which produce cyclic endproducts.

III. α-AMYLASE

α-Amylase (1,4-α-D-glucan glucanohydorolase EC 3.2.1.1), which catalyzes hydrolysis of the α-1,4-glucosidic linkages of starch, is widespread among microbes. It is an endoamylase which liberates poly- and oligosaccharide chains of varying lengths. Dextrinization of the substrate is accompanied by rapid loss of viscosity of the substrate solution. α-Amylases are divided into two categories according to degree of hydrolysis of substrate.[23] Saccharifying α-amylases hydrolyze 50 to 60% and liquefying enzymes about 30 to 40% of starch. Although this division cannot be taken as absolute, it is widely used to describe

the properties of α-amylases. The term saccharogenic amylase, used sometimes, usually denotes glucoamylase.

Specificity of α-amylases for α-1,4-glucosidic bonds is not absolute. A few α-amylases can degrade some α-1,6-glucosidic bonds as well,[24] but the rates of these reactions are much lower than those for 1,4-bonds. Endproducts of amylolysis have an α-configuration at the C_1. Commercial applications of α-amylase are numerous. Probably the largest volume is used for thinning of starch in the liquefaction process in the sugar, alcohol, and brewing industries. α-Amylases are used in desizing of fabrics, in the baking industry, in production of adhesives, pharmaceuticals, and detergents, in sewage treatment, and in animal feed.

A. GENETICS OF α-AMYLASE

Genetic studies of α-amylase have been carried out mostly with genus *Bacillus*, especially with *Bacillus subtilis*. The gene coding for α-amylase, *amyE*, is situated on the circular chromosomal map of *B. subtilis* in the region 25°, where there are the mapped genes *tmrA*, *amyR*, *amyE*, *azc*, *serR*, *tmrB*, and *aroI*.[25] Related *Bacillus* species have allelic α-amylase genes which can be transferred between strains.[26] The *amyE* gene is closely linked to regulatory element *amyR*,[27] originally designated *amyH*,[28] which has an effect on α-amylase production, but not on the specific activity of the enzyme.[29]

Yamaguchi et al.[27] transformed *B. subtilis* 6160 with chromosomal DNA from *Bacillus natto* IAM 1212, which produces 5 times more α-amylase than *B. subtilis* 6160. Enzymes from these strains have different properties.[27] One tenth of the transformants producing high amounts of amylase have properties of *B. subtilis* amylase and the rest those of *B. natto*.[27] In one transformant, *B. subtilis* NA64, which produces α-amylase that resembles *B. subtilis* enzyme,[30,31] the α-amylase structural gene originates from *B. subtilis*. The *amyR* region and surroundings having an effect on productivity are from *B. natto*.[32] In another transformant, *B. subtilis* NA20, the high amount of enzyme is synthesized from a hybrid gene which is mostly *amyE* of *B. natto*.[32] The physicochemical properties of the enzyme are near those for amylase of *B. natto*.[30] Hybrid protein nature of the *B. subtilis* NA20 enzyme is seen clearly from its molecular weight of 42,000[30] which is intermediate between the molecular weights of amylases from *B. subtilis* and *B. natto*, which are 55,000 and 34,000, respectively.[30]

B. subtilis NA20 and NA64 produce more α-amylase than the parental *B. subtilis* 6160 strain because they have the *amyR2* region from *B. natto* in front of the structural gene.[30] The allelic region is called *amyR1 in B. subtilis*[30] and *amyR3* in *B. subtilis* var. *amylosaccharitícus*.[33] *amyR3* increased amylase production in *B. subtilis* about three- to fivefold,[33] suggesting phenotypical similarity of *amyR3* and *amyR2*.

Nicholson and Chambliss propose that also *gra-10*[34] and *gra-5*[35] are alleles of *amyR1*. These mutants and *amyR2* cause catabolite repression-resistance of α-amylase synthesis.[35] They propose that the sequence responsible for *amyR* function is located 5′ to the nucleotide-115 of the *amyE* gene.[36] These factors result from one G-C to A-C transition of 5 bp downstream from the transcription initiation site.[37] The amount of α-amylase produced correlated with the amount of mRNA,[37] thus, the α-amylase production is regulated on transcriptional level. It is suggested that the regulatory *amyE*-binding protein has an analogous mechanism as factors of *cis*-acting regulatory loci in *E. coli*.[37]

An inverted repeat structure from *B. natto* preceding the *B. subtilis amyE* gene confers increased α-amylase production.[38] This AT-rich region is different from the otherwise similar upstream region of *amyR1*.[37] An inverted repeat structure is present to the 5′ side of *Bacillus amyloliquefaciens* amylase gene and functions as a transcription termination signal of an upstream operon.[39] Removal of this region reduced α-amylase production level in *B. subtilis* by 70%.[40] The presence of *amyR2* region of *B. subtilis* NA64 in plasmid preceding the *E. coli* β-lactamase gene enhanced transcription activity approximately sixfold.[40] These data suggest that the *amyR* and *gra* functions could be transcription termination signals which

TABLE 1
Stimulating Effect of Various Genes on α-Amylase Production in *Bacillus Subtilis*

Gene	Fold stimulation of production: α-Amylase	Protease	Ref.
A-2	6		42
amyR1	1	1	30
amyR2	4—5	1	30
amyR3	4—5	1	33
amyS	2—3	1	43
C-108	5	80	42
gra-5	5—10		35
gra-10	5—10		34
papM118	2—3	10—20	43
papS1	2—3	5—10	43
tmrB7	5—7	1	44, 45

prevent α-amylase production by reducing readthrough from an upstream operon. The upstream operon in *B. amyloliquefaciens* produces three polypeptides, the functions of which are unknown.[40] It has been postulated that the palindromic sequence of the *amyR2* is a kind of receptor for factors affecting enzyme production such as *sacU*, *pap*, and *sacQ* gene products.[41] The precise site and function of the *amyR* region has not been determined, but it can be thought to be a region responsible for several functions, including regulation and promoter function and preceding the *amyE* gene.

Many genes and their products are involved in *B. subtilis* α-amylase production. Some mutations having an effect on α-amylase production in *B. subtilis* are presented in Table 1. There is no precise evidence how most of these mutations affect α-amylase production. *sacU*^{h},[46,47] *amyB*,[48] and *pap*[49,50] have a pleiotropic effect. They are phenotypically identical and situated in the same locus.[47] Cells containing any of these mutations produce high amounts of extracellular proteins, e.g., α-amylase and proteases, are poorly transformable, and deficient of flagellae.[47,51] Factor *pap* causes filamentous cells which lack one membrane protein component.[49]

The *sacU* gene may play a regulatory role in the expression of *div-34'*, which has an effect on sporulation.[52] It is assumed that increased exoenzyme production by the *sacU*^{h} mutant originates from its ability to initiate sporulation-associated processes even in the presence of excess nutrients.[52] Exoenzyme secretion and sporulation events are generally linked phenomena in *Bacilli*. Most *Bacillus* strains produce α-amylase at maximum rates in late logarithmic and stationary phases.

Another factor, *sacQ*, having a pleiotropic effect on production of levansucrase and proteases, has also been cloned.[53] The same kind of gene has been cloned from *Bacillus licheniformis*.[55] The *sacQ* gene of *B. licheniformis* gives rise to a hypersecretion phenotype.[55] It affects also α-amylase production when cloned in a multicopy plamid.[55] The target site of levansucrase regulation by *B. licheniformis sacQ* is at a fragment in the 5' noncoding region of *sacB* gene.[55]

Factors *amyS1* and *papS1* from *B. subtilis* var. *amylosaccharitiicus* encode hyperproduction of α-amylase and α-amylase plus protease together, respectively.[43] Some mutants conferring resistance to antibiotics have an effect on exoenzyme production also. *tmrA7*, which causes tunicamycin resistance, increases specifically α-amylase production in *B. subtilis*.[44] The *tmrA* locus precedes the *amyR* region, whereas, other tunicamycin mutants, which do not have an effect on α-amylase production, map in *tmrB* near *aroI* gene.[45] In the *tmrA* mutant, a 16.3-kb fragment containing the *amyE* and *tmrB* regions is repeated tandemly

approximately 10 times in the chromosome of *B. subtilis*.[56] Thus, amplification of the *amyE* and *tmrB* genes results in increased amylase production and tunicamycin resistance. The DNA inducing gene amplification was almost solely in the 1.6-kb fragment, which is composed of the neighboring sequence of junction region of the amplified fragments.[58] It has been suggested that the region might facilitate unequal recombination between replicating sister chromatids in transformation.[58] The increased α-amylase production in *B. subtilis* 2633 was shown to originate from multiple copies of the *amyE* gene in the chromosome.[59]

D-cycloserine and ampicillin resistance causing elements, C-108 and A-2, increase α-amylase production by five- and sixfold, respectively,[42] but the genetic bases of these effects have not been studied. When transformed into the same strain, many of these factors have a synergistic effect on α-amylase production. Industrial production strains have been developed by successive mutagenizations and transformations. As an example, *B. subtilis* PP13, which presumably contains factors *amyR3*, *amyS*, *papS1*, *tmr*, and *papM118*, produces 250 times more amylase than the parental *B. subtilis* 6160.[43] Over 95% of the protein in the culture medium is α-amylase.[43] Strain T2N26 presumably has genetic elements *amyR*, *tmrA7*, *pap*, *amyB*, *sacU*, C108, and A-2 and produces 1500 to 2000 times more enzyme than the parental *B. subtilis* 6160.[42] Markers of these two strains have not been verified. Many mutations produce high amounts of proteases due to their pleiotropic nature as well. It has not been reported if this has a negative effect on amylase production. For other microbes only limited experimental work has been carried out on α-amylase. α-Amylase genes of *B. amyloliquefaciens*[57,60] and *Bacillus stearothermophilus*[61] have been incorporated by transformation into amylase-negative *B. subtilis* strains. Successive mutagenesis with different agents has been used to enhance production in *B. amyloliquefaciens*,[62] *B. licheniformis*,[63] and *Aspergillus niger*.[64] Many constitutively α-amylase-producing strains have been constructed; e.g., *B. licheniformis*,[65] *B. subtilis*,[34] *Schwanniomyces castellii*,[66] *S. occidentalis*,[67] and *Streptomyces hygroscopicus*.[68] Mutation in the *exo-1* locus of *Neuropora crassa* confers hyperproduction of several exoenzymes, including α-amylase and glucoamylase, as well as alterations in the cell wall.[69,70]

Regulation of α-amylase production is poorly understood, but several phenomena are related to regulation; e.g., temporal activation,[34,71] catabolite repression,[72,73] induction,[74,75] and mRNA stability.[76,77] Priest[71] has reviewed these factors. In *Aspergillus awamori*, α-amylase synthesis is translationally controlled.[78] Genetic elements affecting α-amylase production in *B. subtilis* have been studied in relation to cloned α-amylase genes. Production of *B. amyloliquefaciens* amylase cloned in phage Φ3T was increased by *papM*, *papS*, and *amyS*, whereas, *amyR2* and *tmr* stimulated production of *B. subtilis*-type amylase production of the host only.[79] Production of *B. amyloliquefaciens* amylase from a multicopy plasmid in *B. subtilis* was not affected by any of the factors *amyR1*, *amyR2*, *amyR3*, *amyS*, *papS*, and *pap*.[80] It is supposed that at least *amyR* elements closely linked to *amyE* do not function in *trans* configuration.[80]

B. CLONING OF α-AMYLASE GENE

α-Amylase was one of the first proteins adopted for molecular biological studies for several reasons: (1) easy screening assays exist; (2) amylase-negative strains are available; and (3) the genetics, protein production, and fermentation technology of α-amylase in *B. subtilis* is well known. The *Bacillus* structural gene *amyE*, its promoter, and a regulatory element, *amyR*, have been cloned from several strains (see Table 2).

1. Donors of Genes

Donors of the genes have been mainly *Bacillus* strains, with the exceptions of *Aeromonas hydrophila*,[81] *Dictyoglomus thermophila*,[120] *E. coli*,[121] *Saccharomycopsis fibuligera*,[122] *S. hygroscopicus*,[123,124] and *Streptomyces limosus*.[125] The strain used by Cornelis et al.[92] was

TABLE 2
Cloning of α-Amylase Genes

Donor	Plasmid	Vector	Host	Size of insert	Location[a]	Length of signal peptide	Miscellaneous
eromonas hydrophila JMP636	pJP3101	pUC12	*Escherichia coli*	2.1	P		Sequenced; potential signal sequence of 21 residues
acillus sp.	pVC102	pPL603b	*Bacillus subtilis*		E		Produces 30 times more than parental strain
acillus sp. 707	pTUE306	pBR322	*E. coli*	2.5	P		Sequenced; 30—35% of the activity leaks from *E. coli*
	pTUB812	pUB110	*B. subtilis*	2.5	E	33	
acillus amyloliquefaciens sp.		ϕ105	*B. subtilis*	2.3			
. amyloliquefaciens	pGX2509	pBD64	*B. subtilis*				
	pGX1419	pGX1901	*Brevibacterium lactofermentum*				Produces small amount of cross-reacting material
. amyloliquefaciens ATCC 23844	pAAM5		*Saccharomyces cerevisiae*	2.2			Yeast *ADH1* promoter essential for expression
. amyloliquefaciens H	ϕ3T Amy$^+$	ϕ3T	*B. subtilis*		E		
. amyloliquefaciens E 18	pKTH10	pUB110	*B. subtilis*	2.3	E	31	Same strain as VTT197; sequenced; produces 500 times more than wild-type strain; copy number about 50
acillus coagulans	pAMY2	pBR322	*E. coli*	3.31	P		
acillus licheniformis	pEAA	pHV33	*B. subtilis*				
. licheniformis ATCC 6598	pJ01	pBR322	*E. coli*	3.3	P		Orientation has no effect on production
	pJ04	pUB110	*B. subtilis*	3.3	E		
	pMA2	pKT230	*Pseudomonas aeroginosa*	3.3	P		
. licheniformis ATCC 14580	pKTH1503	pBR322	*E. coli*	5.3			Cells are leaking enzyme to medium. 5′ end sequenced

B. licheniformis ATCC 27811	pTN1	pBR322	*E. coli*	2.5			Production almost the same in spite of orientation; sequenced; same strain as 584	9
	pTN2	pBR322	*E. coli*	2.1				
B. licheniformis FD02	pSA 36-2	pBD64	*B. subtilis*	2.55	E	29	Produces 20 times more than the donor in spite of orientation; 5′ end sequenced	9
	pSA 36-3	pBD64	*B. subtilis*	2.55	E			
B. licheniformis NCIB 8061	pBR322BL	pBR322	*E. coli*	2.3	P	29	Sequenced	1
	pBS42BL	pBS42	*B. subtilis*	2.3				
B. licheniformis RP01	pRP1	pBD64	*B. subtilis*	3.5	E			1
B. licheniformis TY 002	pNTK3	pBR322	*E. coli*	2.7	P			1
	pSNK1	pUB110	*B. subtilis*	2.7				
Bacillus megaterium	pAMY100		*B. subtilis*	2.1			Sequenced; putative signal sequence of 27 residues	1
Bacillus stearothermophilus		pBR322	*E. coli*	5.4			Plasmid-borne gene	1
B. stearothermophilus	pUC13BS	pUC13	*E. coli*	1.6	P	34	Sequenced	1
	pBS42BS	pBS43	*B. subtilis*	1.6				
B. stearothermophilus 799	pH799-9	pBR322	*E. coli*					1
B. stearothermophilus A-10	p69Amy1	p69A	*S. cerevisiae*	1.7			Expression under yeast α-factor	1
B. stearothermophilus ATCC 12980	pCSS1	pUC8	*E. coli*	1.9	P	34	Sequenced; processed at two sites in *E. coli*	1
	pCSS502	pPL608	*B. subtilis*	1.9	E	34		
S. stearothermophilus ATCC31199	pamy7	pCA43	*S. carnosus*	5.4	E		Plasmid-borne gene	1
	pamy7	pCA43	*S. aureus*	5.4	E			
	pamy7	pCA43	*S. hyicus*	5.4	E			
	pamy7	pCA43	*S. xylosus*	5.4	E			
	pamy7	pCA43	*S. epidermis*	5.4	E			
S. stearothermophilus CU21	pAT5	pTB53	*B. subtilis*	7.27	E	34	Sequenced	1
	pAT9	pTB90	*B. stearothermophilus*	7.27	E			
	pBR,2OA	pBR322	*E. coli*	8.0				
S. stearothermophilus DY-5	pHI301	pBR322	*E. coli*	3.1	P	34	Sequenced	1
	pBAM1	pUB110	*B. brevis*	3.1	E	34		
	pBAM1	pUB110	*B. subtilis*	3.1	E	34		
S. subtilis 168		pBR322	*E. coli*			32	Sequenced	1

TABLE 2 (continued)
Cloning of α-Amylase Genes

Donor	Plasmid	Vector	Host	Size of insert	Location[a]	Length of of signal peptide	Miscellaneous	
. *subtilis* 2633	pAM26	pHY300PLK	*E. coli*	2.3	P		Sequenced	5
	pAM26	pHY300PLK	*B. subtilis*	2.3				
. *subtilis* N7	pTUB101	pUB110	*B. subtilis*	2.5	E	41	Sequenced. Donor transformed with *B. natto* DNA	1
. *subtilis* NA64	pTUB4	pUB110	*B. subtilis*	2.3	E	41	Sequenced partly from hybrid	3
ictyoglomus thermophilum H-6-12	pDT901	pYEJ001	*E. coli*	2.6	C	1	Sequenced	1
scherichia coli MC 4100	pSF1707-8	pBR322	*E. coli*	4	P			1
accharomycopsis fibuligera HUT 7212	pSf 2	pYI1	*S. cerevisiae*	3.8	E			1
treptomyces hygroscopicus	pPOD1039	pIJ702	*S. lividans*	2.8	E		Produces α-amylase 35% out of extracellular protein	1
. *hygroscopicus* AA69-4	pMS120	pUC12	*E. coli*	2.7	P	30	Donor strain mutated from	1
	pMS101		*S. lividans*	2			strain SF-1084; sequenced	
. *limosus* ATCC 19778	pSYC1318	pIJ941	*S. lividans*	2.5	E	28	Sequenced	1

(C) cellular; (E) extracellular; and (I) intracellular enzymes.

typed as *Bacillus coagulans* or *B. amyloliquefaciens*. Yamane et al.[118] claim that they have cloned the *B. natto* type amylase gene from *B. subtilis* N7, which was isolated following transformation by *B. natto* DNA.[118] However, the cloned gene presumably does not contain the whole *B. natto* structural gene, based on protein molecular weights consideration. The molecular weight of *B. natto* amylase is 36,000,[30] while that of the *B. subtilis* N7 enzyme and the protein produced from its cloned gene is 52,678.[118] It is thus likely that *B. subtilis* N7 arose by formation of *B. natto amyR2-B. subtilis amyE* hybrid in the transformant. Cloned genes have been of chromosomal origin. However, two *B. stearothermophilus* strains contain plasmid-borne α-amylase genes.[104,109] Copy number of 24-kb plasmid containing α-amylase gene is about ten.[104] The plasmid-borne gene does not hybridize with chromosomal DNA in Southern hybridization,[104] while the gene from *B. stearothermophilus* ATCC 12980 does hybridize with chromosomal DNA.[108] *B. stearothermophilus* species are known to be quite heterogeneous. Some strains have greater evolutionary distances than those between several species of genus *Bacillus*.[105] It is possible that the *B. stearothermophilus* strains having plasmid-borne α-amylase genes are only remotely related to other *B. stearothermophilus* strains and should possibly be reclassified. α-Amylase gene might originally have been plasmid-borne in *B. stearothermophilus*, but it has been integrated into genome in most strains.

Cloning of the first amylase was performed with temperate *B. subtilis* Φ3T phage.[88] The phage DNA and the cloned *B. amyloliquefaciens* amylase gene integrated near the lysogen attachment site in the vicinity of the *thyP3* locus, far from the *amyE* region.[88] Subsequently, plasmid vectors have been employed (see Table 2), but phages have been used several times to prepare genomic libraries from which amylase genes have been isolated by screening. In addition to the most frequently used *E. coli* and *B. subtilis*, for which there are the most advanced genetic methods, host organisms have included *Bacillus brevis*,[113] *B. stearothermophilus*,[110] *Brevibacterium lactofermentum*,[86] *Pseudomonas aeruginosa*,[95] *Saccharomyces cerevisiae*,[87,106,122] and some *Streptomyces* strains.[109,123,125] Most frequently used have been pBR322- or pUC-based plasmids for cloning into *E. coli* and pUB110-based plasmids for cloning into *B. subtilis*.

2. Nucleotide Sequences of α-Amylase Genes

Quite a number of α-amylase genes have been sequenced; these include those from *A. hydrophila*,[81] *Bacillus* sp.,[85] *B. amyloliquefaciens*,[91] *B. licheniformis*,[97,100] *Bacillus megaterium*,[103] *B. stearothermophilus*,[99,107,111,114] *B. subtilis*,[116-118] *D. thermophila*,[120] *S. hygroscopicus*,[124] and *S. limosus*.[125] The main part of the DNA sequence for *B. subtilis* NA64 amylase[38] has been sequenced, as well as the 5′ terminus of sequences for *B. licheniformis* FDO2[99] and ATCC 14580[96] genes.

The sequenced amylase genes contain typical −35 and −10 promoter regions for recognition by vegetative δ-factor of *Bacillus* and also contain signal peptide coding regions. Though amylase genes from near relatives are quite similar (e.g., the genes from *B. stearothermophilus* and *B. licheniformis* have 59 and 62% similarity in nucleotide and deduced polypeptide sequences, respectively[100]), α-amylases from mammals, plants, and microbes differ markedly. Genes for *Bacillus* α-amylases are monocistronic because DNA sequences contain inverted repeat sequences which can function as transcription termination signals before and after the sequence for structural gene, e.g., in *B. amyloliquefaciens*,[91] *B. licheniformis*,[97] *B. stearothermophilus*,[111] or after the sequence, as in *B. subtilis*.[116] Gene amplification occurs in two *B. subtilis* strains, which have been extensively mutated to produce high amount of α-amylase.[57,58] In these strains increased α-amylase production correlates with increased gene dosage. In *B. subtilis*, the *amyR2* regulatory region located 100 to 300 nucleotides upstream from *amyE*[103] has been shown to contain an AT-rich inverted repeat structure,[38] which could terminate transcription originating from promoter further upstream.

3. Signal Peptide and Amino Acid Sequences of α-Amylases

Bacillus α-amylases are extracellular enzymes, and they are also secreted to culture medium when expressed from cloned genes in *Bacillus, Staphylococcus,* and *Streptomyces* strains (see Table 2). In general, the amino terminal signal peptide of unprocessed protein is removed when the protein is secreted.[126] For the sequenced α-amylases, the length of the signal peptide varies from 29 to 41 amino acids (see Table 2). The precise cleavage site can be determined by amino terminal amino acid sequencing, and this has been done for α-amylases from *Bacillus* sp.,[85] *B. amyloliquefaciens* E18,[89] *B. stearothermophilus* ATCC 12980,[107] CU21[111] and DY-5,[114] *B. subtilis* NA68 (characterized from *B. subtilis* YY88[127]), *S. hygroscopicus* AA69-4,[124] and *S. limosus.*[125] Signal peptides of cloned *B. stearothermophilus* DY-5 and CU21 α-amylases are processed at the same site in *E. coli* and the donor strain.[111,114] The signal peptide of the *B. stearothermophilus* ATCC 12980 enzyme is processed in the same way in *B. stearothermophilus* and *B. subtilis,*[108] but at two closely located sites at almost equal frequency in *E. coli.*[107] One of these sites is the same as in the *Bacillus* strains. It has been postulated that the differences in *B. stearothermophilus* α-amylase processing arise from *E. coli* signal peptidase for which this signal peptide is longer than in *E. coli* proteins.[107] The enzyme cuts from the first possible site but also from the actual cleavage site just three residues further.[107] *A. hydrophila, B. licheniformis,* and *B. megaterium* enzymes have putative signal peptide of 21, 29, and 27 residues, respectively,[81,99,103] but have not been verified from produced proteins. Processing of *D. hydrophilum* α-amylase in *E. coli* leads to a loss of just one residue from N-terminus.[120]

In addition to several amino acid sequences deduced from nucleotide sequences and verified by sequencing the amino terminus, amino acid sequence of *Aspergillus oryzae* α-amylase has been determined.[128] Partial amino acid sequences are known for amylases from *B. subtilis* var. *amylosacchariticus,*[129-132] *B. amyloliquefaciens,*[133-135] and *B. licheniformis* ERYR9.[136] α-Amylase sequences contain three[137] or four[138,139] conserved regions (see Figure 2), and these regions have been proposed to be essential for the function of α-amylase because they are aligned and spaced at similar intervals along the proteins. These regions presumably form the active center, the substrate-binding site, and the site for binding the stabilizing calcium ion. The region nearest to the amino terminus shows homology with the calcium-binding site of calmodulin, troponin C, and myosin light chain.[137] The fourth conserved region[138,139] is just 4 amino acids in length, and only one of them was invariant in 11 sequences; thus, the significance of this region is not known. According to comparison with the *A. oryzae* α-amylase sequence, whose refined three-dimensional structure is known,[140] the amino acids of conserved regions contribute to activity, substrate, and Ca-binding.

4. Expression of Cloned α-Amylase Genes

Cloned α-amylases are secreted from *Bacillus, Pseudomonas,* and *Staphylococcus* strains (see Table 2). In *E. coli,* α-amylase is usually transported into periplasmic space (see Table 2). However, the *B. stearothermophilus* enzyme synthesized in *E. coli* can be released from cells, probably due to a stress response which can be related to activation of autolytic activities.[141] Cell lysis is not necessary for this phenomenon. Overexpression of α-amylase in *E. coli* causes release of *E. coli* periplasmic proteins into the extracellular space.[141]

The α-amylase levels vary greatly depending on clones and hosts (Table 2). Some clones produce the same amount regardless of orientation of gene in plasmid.[94,97,98] Most of the work to date has used basic cloning vectors which are not necessarily the best for large-scale production. Most cloned α-amylase genes are expressed from their own promoter, especially in *E. coli, B. subtilis,* and *Streptomyces* strains (see Table 2). Expression of the cloned *B. amyloliquefaciens* α-amylase gene in *S. cerevisiae* requires yeast *ADH1* promoter.[87] This is reasonable since eukaryotic cells have different expression machinery, e.g., different recognition sites, their spacing, and factors of enzymes than prokaryotic species. *B. stear-*

	I		II	
TAA	117	DVVANH 122	202	GLRIDTVKH 210
BStA	101	DVVFDH 106	230	GFRLDAVKH 238
BAA	98	DVVLNH 103	227	GFRIDAAKH 235
BLA	100	DVVINH 105	227	GFRLDAVKH 235
BME	109	DLVVNH 114	202	GFRLDAAKH 210
BSP	103	DVVMNH 108	233	GFRIDAVKH 241
BSU	107	DAVINH 112	181	GFRFDAAKH 190
SHY	88	DAVVNH 93	170	GFRIDAAKH 178

	III		IV	
TAA	229	GEVLD 233	292	FVENHD 297
BStA	263	GEYWS 267	326	FVDNHD 332
BAA	260	AEYWQ 264	323	FVENHD 328
BLA	260	AEYWQ 264	323	FVDNHD 328
BME	246	GEVWD 250	308	FLTNHD 313
BSP	265	VEFWK 270	329	FVDNHD 334
BSU	217	GEILQ 221	274	WVESHD 279
SHY	199	QEVIY 203	257	FVDNWD 262

FIGURE 2. Conserved regions (I to IV) of some microbial α-amylases. TAA = *Aspergillus oryzae*;[128] BStA = *Bacillus stearothermophilus*;[107] BAA = *B. amyloliquefaciens*;[91] BLA = *B. licheniformis*;[97] BME = *B. megaterium*;[103] BSP = *Bacillus* sp.;[85] BSU = *B. subtilis*;[116] and SHY = *Saccharomycopsis hygroscopicus*[124] enzymes.

othermophilus α-amylase gene requires yeast promoter and signal peptide from yeast-mating pheromone α-factor for expression and export in *S. cerevisiae*.[106] *S. fibuligera* α-amylase gene seems to be expressed from its own promoter in yeast.[122]

Stability of constructed plasmids has also been a problem. Plasmid instability can be of segregational or structural type. Segregational instability results from improper partitioning of plasmids to daughter cells and leads to a remarkable loss of the whole plasmid. Structural instability designates rearrangements in plasmids, most frequently by deletions. Some *E. coli* constructions are structurally unstable,[92,93,96,145] while others (plasmids pHI303[98] and

pKTH1503[96]) are stable. Segregational instability can be decreased by using antibiotics for which plasmids confer resistance. Plasmid pSA36-2 is not stably maintained in *B. subtilis* without antibiotic pressure,[95] and *B. subtilis* (pJO4) segregates the plasmid in five generations when grown without kanamycin.[94] Plasmids pTUB812 and pTUE306 were totally maintained after 50 generations in *B. subtilis* and *E. coli*, respectively.[84] Plasmid instability of cloned *B. amyloliquefaciens* α-amylase in *B. subtilis* is related to high expression of the cloned gene in the *sacU9* strain.[142] Plasmid instability in *B. subtilis* is probably associated with postexponential phases of growth.[83]

Plasmids carrying *B. amyloliquefaciens* α-amylase gene in *S. cerevisiae* are stable per se, but the strains exhibited segregational instability, which decreased the level of produced enzyme, especially when grown on nonselective medium. To overcome problems with plasmid instability, the genes can be integrated in the chromosome of producing organisms. The genes are stably maintained, and it is possible to increase gene dosage by amplification. Two *B. amyloliquefaciens* genes have been integrated in the genome of *B. subtilis*.[144,145] Eight α-amylase genes could be found from cells producing 8 times more α-amylase than the parental strain.[145] The amounts of produced enzyme were comparable to production from multicopy plasmid.[145]

α-Amylase regulatory and signal sequences have been used to construct expression/secretion vectors.[146,147] These have been used to express in *B. subtilis*, *E. coli* β-lactamase,[146-148] interferon,[149,150] α-amylase,[151] and virus membrane glycoprotein.[152] Cloned α-amylase genes have been used as model systems in several studies, and they have also been used for commercial applications. However, there still remain many problems concerning stability, expression, and secretion. Gray et al.[100] constructed hybrids of *B. stearothermophilus* and *B. licheniformis* enzymes, which have different properties from the parental enzymes in terms of specific activity and temperature stability. The former enzyme has higher specific activity, but lower thermostability, than the latter.[100] The hybrid proteins have intermediate properties.[100]

C. ENZYMES OF MICROBES GROWN UNDER EXTREME CONDITIONS

There is quite a clear correlation between the conditions found in the environmental niche occupied by a microbe and the properties of its enzymes. Thermophilic organisms produce enzymes which are, in general, more thermostable than enzymes produced by mesophilic organisms. Enzymes of halophilic organisms require salts to be active and are most active in quite high salt concentrations which inhibit other amylases. Alkalo- and acidophilic microbes produce enzymes which are most active at extreme pH values.

1. Halophiles

Halophilic bacteria can be divided into two groups: moderate halophiles, which grow in salt concentrations of 2 to 20%, and extreme halophiles, which require more than 10% salt for growth.[153] In one survey amylolytic activity was found from 9 out of 49 extreme halophiles.[153] Amylases have been studied from only five halophilic microorganisms; *Acinetobacter* sp.,[154] *Halobacterium* sp.,[155] *Halobacterium halobium*,[156] *Micrococcus halobius*,[157] and *Micrococcus varians* subsp. *halophilus*.[158] *H. halobium* and *Halobacterium* sp. are extreme halophiles and the others are moderately halophilic.

These enzymes require the presence of Na, K, and Ca ions for activity and stability. Amylase production is increased by the presence of starch or maltose in *Acinetobacter*, and optimal production occurs in 2 *M* NaCl or 1 *M* KCl.[154] Starch-inducible α-amylase production in *M. halobius* is maximal at 1 *M* NaCl.[157,159] *M. varians* produces α-amylase maximally in 2 *M* NaCl with maltose as inducer.[158] The salt requirement for amylase activity from moderately thermophilic and halophilic *Acinetobacter* is higher than that from the extreme halophile *H. halobium*.[154] The properties of *Acinetobacter* amylase are dependent on the

presence or absence of calcium.[154] Purified *M. halobius* enzyme requires 0.25 *M* NaCl or 0.75 *M* KCl for maximal activity.[157] *Acinetobacter* and *M. varians* produce two amylases, and, except for molecular weight, these isoenzymes have very similar properties.[154,155]

2. Alkalophiles

Only a few alkalophilic amylase-producing strains have been studied.[160-162] The pH optima for growth of *Bacillus* strains are in the alkaline region, 10.5 for *Bacillus* No A-40-2,[160] about 10 for strains studied by Yamamoto et al.,[161] and 9.5 for *Bacillus* sp.[162] Likewise, the pH optima of the amylases produced by these strains lie in the range of 9 to 10.5 (see Table 3). Some of these crude enzyme preparations have two pH optima, which may indicate the presence of additional amylolytic activities. Amylase activity from *Bacillus* sp. has three pH optima.[162] The identities of these enzymes have not been elucidated, but they may be α-amylases. Alkalophilic amylases exhibit a broad pH stability (see Table 3). *B. licheniformis* CUMC 305 produces α-amylase having a pH optimum of 9.5,[163] but the strain grows optimally at pH 6.5.[164] The temperature optimum of this enzyme is exceptionally high, 90°C.[163]

3. Acidophiles

Enzymes from acidophilic *Bacillus* strains have pH optima at 2 to 4.5 (see Table 3). The pH optimum is 3.0 to 4.0 for growth of *Bacillus* sp.[165] and for *Bacillus acidocaldarius* 104-1A[166] and is 3.5 for *B. acidocaldarius* Agnano 101.[167] Alkalophilic *Bacillus* strains No. 13 and 17-1 produce amylases which have a pH optimum at 4.5.[161] These amylases are quite thermostable, and they have a temperature optimum for activity in the range 60 to 70°C, while the bacteria grow in temperatures of 55 to 60°C (see Table 3). Enzyme from *B. acidocaldarius* A-2 tolerates 90°C at pH 4.5 and 70°C at pH 3.5 for 15 min without remarkable loss of activity.[168]

4. Thermophiles

Of all the α-amylases having extreme properties, the thermostable enzymes are the most studied due to both fundamental interest on the basis of thermostability and to the numerous industrial applications of thermostable enzymes. Thermophiles can be divided into three categories according to Amelunxen and Murdock:[169] (1) obligate or strict thermophiles, which have growth optima ranging from 65 to 70°C but showing no growth below 40 to 42°C; (2) facultative thermophiles, with optimum growth temperature between 50 and 65°C, but also capable of reproducing at temperatures below 30°C; and (3) thermotolerant bacteria with growth maxima at 45 to 50°C and showing growth below 30°C. Because systematic studies are rare among amylase-producing microbes, we consider here thermophilic microbes which have been cultivated at 55°C or higher; *D. thermophilum,* which grows optimally at 78°C,[120] is thus far the most thermophilic α-amylase-producing microorganism studied.

α-Amylases of thermophilic organisms have quite high temperature optima and are also thermostable (see Table 3). Some mesophilic organisms, e.g., *B. licheniformis* CUMC 305 and NCIB 6346, produce enzymes whose temperature optima are in the range 70 to 90°C.[163,170] The amylase of *B. licheniformis* CUMC 305 has 45% of its maximal activity at 110°C.[163] Many organisms have lower temperature for optimal growth than for the maximum activity of the α-amylase they produce.

Campbell[171] indicated that α-amylases of *B. coagulans* 43P and *B. stearothermophilus* 1518 have temperature optima dependent on growth temperature, but these results could not be reproduced,[172] and it was suggested that the presence of two variants caused the original result.[172] Differences in heat stabilities of *B. stearothermophilus* enzyme produced at 37 and 55°C[173] are not significant.[172] *B. stearothermophilus* Donk BS-1 produces two amylolytic enzymes at 55°C and only one at 43°C.[174] Enzymes "55°C I" and "43°C" are identical,

whereas "55°C amylase II" has a different thermostability and molecular weight.[175] The "55°C enzyme II" is likely to be a different amylolytic enzyme and not a proteolytic cleavage product of the "55°C enzyme I".[175]

D. α-AMYLASES OF *ACTINOMYCETES* AND FUNGI

Studies on bacterial α-amylases have been carried out mostly within the genus *Bacillus*, but a few reports deal with actinomycetal enzymes. Of these, *Thermoactinomyces* sp. 15, has the highest temperature optimum α-amylase, 80°C.[176] It is remarkably heat-stable; exposure to 100°C inactivates only 20% of original activity in 30 min.[176] Properties of enzymes from *Actinomycetes* resemble usually those of other amylases. Some enzymes produce high amounts of certain oligosaccharides from starch (see also Section IV). The enzyme of *Thermomonospora curvata* produces mainly maltotetraose and maltopentaose.[177] *Thermoactinomyces* spp. are presented here, although their classification to *Actinomycetes* is questionable. Many of their properties are near those for *Bacillus* and *Clostridium* strains and dissimilar from *Euactinomycetes*.[178]

Amylolytic enzymes are of widespread occurrence in fungi (see Tables 3 and 9), which often produce several activities into medium, mainly α-amylase and glucoamylases. Amylolytic activities have been screened from thermophilic fungi,[179] thermotolerant and thermophilic fungi,[180] *Bacidiomycetes*,[181] *Aureobasidium pullulans*,[182] *Lipomyces*, *Schwanniomyces*,[183] and *Paecilomyces* strains,[184] and from yeasts and yeast-like organisms.[185-191] Even in the more detailed studies, the action pattern of enzymes has usually not been studied, so that the identity of the produced enzymes is not known, e.g., amylases of *Talaromyces emersonii*[192] and *Fusarium vasinfectum*.[193] *F. scirpi* and *F. oxyporum* produce two amylolytic activities referred to as "dextrinizing" and "saccharifying."[194] The saccharifying activity may be glucoamylase. *A. oryzae* EI 212 produces α- and β-amylases.[195] Also, since action patterns and mutarotation of endproducts have not been studied, it is possible that the saccharifying activity is glucoamylase.

Fungi have been divided in this context into yeasts and other fungi because yeasts compose a well-studied and distinct group. Classification was made according to Barnett et al.[196] Properties of fungal α-amylases are quite similar to bacterial enzymes. They are usually less thermostable than bacterial enzymes, but have a somewhat wider range of pH stability. The growth temperatures of the fungi studied are at 45°C or below (see Table 3). *Aspergillus* enzymes have been used for a long time in industrial applications. Taka-amylase A of *A. oryzae* is the most studied amylolytic enzyme.

All yeast and most of the other fungal α-amylases are extracellular enzymes, which have pH optimum near neutrality or in the acidic range (see Table 3). Temperature optima of yeast amylases are at or below 55°C and are produced at moderately low temperatures, 30°C and below. Molecular weights vary from 38,000 to 62,000.

E. PROPERTIES OF α-AMYLASES

Some physicochemical properties of α-amylases are presented in Table 3. Most of the enzymes are extracellular. Intracellular amylases are found from, besides those mentioned in Table 3, *E. coli*,[330] *Streptococcus bovis*,[331] and *F. vasinfectum*, which has three amylolytic activities.[193] It is possible that the latter has three different enzymes, including glucoamylases, which have quite similar properties. Intracellular and membrane-bound enzymes are identical in *B. acidocaldarius*.[167] It is not known why some strains produce intracellular α-amylases because starch cannot penetrate into cells. Membrane-bound amylases are produced in *Lipomyces starkeyi*[319] and *Bacillus* sp.[198] In *Pichia polymorpha*, one form is tightly bound to membrane, whereas, another can be liberated with extraction.[322] *B. subtilis* YY88 has extracellular and membrane-bound α-amylase.[127] *B. subtilis* NA64 produces extracellular enzyme and three larger membrane bound precursors A, B, and C.[248] The physicochemical

TABLE 3
Properties of α-Amylases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp optimum (°C)	pH stability[b]	Thermostability	K_m (mg/ml)[c]	Mol wt[d]	Isoelectric point	Effect of Ca^{2+}[e]	Inhibitors	Miscellaneous
					Bacterial							
cinetobacter sp. 04-1	30	E	7.0	50—55 (−Ca) 60 (+Ca)	7.0—8.0 50°C, 40 min (−Ca) 7.0—9.0 50°C, 40 min (+Ca)			55,000 (1)				Two isomers; in the absence of Ca and NaCl or KCl, two-temperature optima
	30	E	7.0	50—55 (−Ca) 60 (+Ca)	7.0—8.0 50°C, 40 min (−Ca) 7.0—9.0 50°C, 40 min (+Ca)			65,000 (1)				
acillus sp. 1—1S	65	E	2.0	70	1.0—2.5 65°C, 15 min	80% 60°C, 30 min 6% 70°C, 30 min	1.64	54,000 (1)				Ca^{2+} has hardly any effect; EDTA has no effect
acillus sp. AK-2	50	I	6.0	65			0.84			+	Ag^{+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, K^{+}, Pb^{2+}, EDTA, PCMB	Two enzymes, probably isoenzymes, which have similar properties
acillus sp.		E	4.0; 9.0; 11.0									3 pH-optima; different end products in different pH values
acillus sp. 26-1-5	50	E	6.6	82		100% 40°C, 15 min 30% 100°C, 10 min					Ag^{+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, EDTA, PCMB	Ca^{2+} has no effect
acillus No. A-40-2	37	E	10.0—10.5	55	8.5 50°C, 15 min (+Ca)	100% 50°C, 15 min (+Ca) 60% 50°C, 15 min (−Ca)		70,000 (4)		+	Urea, sodiumlauryl-sulfate, sodium-dodecylbenzene-sulfonate	Saccharifying enzyme; ATCC No. 21592
acillus No. 13	37	E	4.5		6.5—10 50°C, 15 min	73% 55°C, 15 min (+Ca) 48% 55°C, 15 min (−Ca)				+		
acillus No. 17-1	37	E	4.5		6.5—10 50°C, 15 min	97% 55°C, 15 min (+Ca) 87% 55°C, 15 min (−Ca)				+		
acillus No. 27-1	37	E	10.5		7—9 50°C, 15 min	42% 55°C, 15 min (+Ca) 8% 55°C, 15 min (−Ca)				+		ATCC number 21596

TABLE 3 (continued)
Properties of α-Amylases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp optimum (°C)	pH stability[b]	Thermostability	K_m (mg/ml)[c]	Mol wt[d]	Isoelectric point	Effect of Ca^{2+}[e]	Inhibitors	Miscellaneous	Ref.
Bacillus No. 38-2	37	E	4.5; 9.0		5.0—10.5 50°C, 15 min	100% 55°C, 15 min (+Ca) 94% 55°C, 15 min (−Ca)				+			161
Bacillus No. 124-1	37	E	10.5		7—9 50°C, 15 min	22% 55°C, 15 min (+Ca) 6% 55°C, 15 min (−Ca)				+		ATCC number 21593	161
Bacillus No. 135	37	E	4.0—4.5; 10.0		7—9 50°C, 15 min	95% 55°C, 15 min (+Ca) 66% 55°C, 15 min (−Ca)				+		ATCC number 21595	161
Bacillus No. 169	37	E	4.0—4.5; 10.0		7—9 50°C, 15 min	95% 55°C, 15 min (+Ca) 57% 55°C, 15 min (−Ca)				+		ATCC number 21594	161
Bacillus acidocaldarius A-2	50	E	3.5	70	4.0—5.5 80°C, 15 min	100% 90°C, 15 min 18% 95°C, 15 min	1.60	66,000 (1)			Cu^{2+}, Fe^{2+}, Zn^{2+}	$CaCl_2$ and BSA does not stabilize; liquefying enzyme	168
B. acidocaldarius Agnano 101	60	E	3.5	75	1.2—6.5 20°C, 90 min below 4.5 24 h	70% 75°C, 60 min 60% 75°C, 2 h 1.25 (3)	0.8 (1,2)	68,000 (1,3) 57,000 (4)		+		Enzyme is both secreted and bound to cell wall; Also Mg^{2+} stimulates and reactivates dialyzed preparation	167
B. acidocaldarius 104-1A	55	E	4.5	60—63								EDTA inhibites only slightly	199
Bacillus alcalophilus subsp. *halodurans*	37	E	10.5		7—9.5 50°C, 15 min	67% 55°C, 15 min (+Ca) 7% 55°C, 15 min (−Ca)				+		Previously *Bacillus* No. A-59	161
Bacillus amyloliquefaciens	37	E	5.5	50—70	6.0—12.0 4°C, 24 h 12% 60°C, 30 min	65% 50°C, 30 min	6.9	58,000 (1) 60,000 (3)	5.2			Trypsin does not digest; substrate stabilizes	200
B. amyloliquefaciens	37	E	6.0	50—70		57% 60°C, 15 min							201

amyloliquefaciens	37	E	6.0	50—70		57% 60°C, 15 min						
amyloliquefaciens	37	E	6.0	50—70		70% 60°C, 15 min						
amyloliquefaciens B20	30	E				26% 85°C, 60 min(+Ca, Na)						
amyloliquefaciens F	37	E	5.9	45;65	5.9 25°C, 2 h 5—9 4°C, 20 h	100% 35°C, 60 min 50% 80°C, 5 min	3.57	49,297 (5)	5.19		EDTA	Liquefying enzyme, ATCC number 23350
amyloliquefaciens N	37	E	5.9	65	4.5—8.0 25°C, 2 h	100% 35°C, 60 min 88% 25°C, 60 min	2.03	49,134 (5)	5.11 (monomer) 4.91 (dimer)			Same strain as *B. subtilis;* ATCC No. 23845
amyloliquefaciens P	37	E	5.9	65	4.5—8.0 25°C, 2 h	100% 25°C, 60 min 94% 35°, 60 min	3.44	49,627 (5) 5.03 (dimer)	5.19 (monomer)			Produces also two membrane-bound forms
amyloliquefaciens SB	37	E	5.9	45, 65	4.5—8.0 25°C, 2 h	100% 25°C, 60 min 91% 35°C, 60 min	2.63	49,610 (5)	5.09 (monomer) 4.59 (dimer)			
amyloliquefaciens T	37	E	5.9	45, 65	4.5—8.0 25°C, 2 h	100% 25°C, 60 min 85% 35°C, 60 min	2.22	48,109 (5)	5.19 (monomer) 5.03 (dimer)			Same strain as *B. subtilis* N; ATCC No. 23842
acillus caldolyticus DSM405	72	E	5.4	70		70% 80°C, 2 h(+Ca) 5% 90°C, 2 h(−Ca)		*ca.* 10,000 (6)		+		
acillus cereus NY-14	30	E	6.0	55	5.5—12 4°C, 24 h 5.5—10 30°C, 60 min	84% 45°C, 30 min 22% 60°C, 30 min		55,000 (1) 36,000 (4)	6.13	+	Ag^+, Cd^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Zn^{2+}	Produces mainly maltopentaose Iodoacetamide and PCMB do not inhibit
acillus circulans	30	E	7.0	50	6—9 30°C, 3 h	100% 50°C, 10 min (+Ca) 60% 50°C, 10 min (−Ca)			45,000	+	Ag^+, Cu^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}	Produces maltotetraose and -pentaose
circulans 186	56		4.75	65		45% 65°C, 2 h (+Ca) 10% 65°C, 6 h (+Ca)		17,000 (4)		+		
circulans G-6	30	E	8.0	60	5—10 30°C, 3 h	60% 60°C, 10 min (+Ca) 50% 60°C, 10 min (−Ca)	0.065%	76,000 (4)		+	Cu^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+}, Ni^{2+}, Sr^{2+}, Zn^{2+}	Produces mainly maltohexaose

TABLE 3 (continued)
Properties of α-Amylases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp optimum (°C)	pH stability[b]	Thermostability	K_m (mg/ml)[c]	Mol wt[d]	Isoelectric point	Effect of Ca^{2+}[e]	Inhibitors	Miscellaneous	Ref.
Bacillus coagulans 43P	35	E	6.5—8.0	45—55	5.0—9.5	8% 90°C, 60 min				+		$CaCl_2$, KCl, and NaCl-reactivate-dialyzed preparation	171
	55	E	6.5—8.0	60—70	5.0—9.5	94% 90°C, 60 min							
B. coagulans CUMC 512	53	E	7.5—8.5	85	4—10 9.0 30°C, 1 h	95% 60°C, 60 min 16% 80°C, 20 min						Substrate stabilizes	163
B. coagulans No. 109	30	I	6.2	50	6.0—7.3 40°C, 2 h	Below 45°C, 15 mi	1.5 m*M* (1) 4.5 m*M* (6) 4.0 m*M* (7) 2.3 m*M* (8) 1.5 m*M* (9) 10 m*M* (10) 2.8 m*M* (11) 0.47 m*M* (12)	62,000 (1)	5.0		Fe^{2+}, Fe^{3+}, Hg^{2+}	Cyclodextrinase	220
Bacillus licheniformis			7.0	90		100% 70°C, 6 h (+Ca) 10% 70°C, 4 h (−Ca)			5.4			Producer Novo Industri A/S, Denmark; Trademark Termamyl®	221—223
B. licheniformis 584	50	E	5.0—8.0	76	6—11 25°C, 24 h	100% 60°C, 15 min 40% 80°C, 15 min		55,200 (2)				EDTA has only slight effect; Liquefying enzyme ATCC number 27811; FERM-P No. 1038; produces maltopentaose; structural gene has been cloned	97, 224
B. licheniformis ATCC 9945	30	E				100% 85°, 60 min (+Ca, Na)							170

licheniformis LM 1777		E	6—7	85	6.5—10.0 25°C, 24 h	94% 75°C, 60 min (+Ca) 20% 75°C, 15 min (−Ca)	0.8	62,000 (1) 48,700 (3) 23,000 (4)	5.2	+	Al^{3+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, EDTA	Trademark Taka-Therm®, producer Miles Laboratories Inc., U.S., needs only small amount of Ca^{2+}
licheniformis UMC 305	48	E	9.0	90	7—9 30°C, 18 h	91% 70°C, 3 h 71% 80°C, 90 min	1.27 1.8 (1) 1.94 (2) 2.28 (3)	28,000 (1)		+	Ag^{+}, Al^{3+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, PCMB sodiumiodoacetate	Also Na^{+} and Mg^{2+} stimulate; Cu^{2+} and Fe^{2+} activate preparations inactivated with EDTA treatment
licheniformis D02	37	E		93		97% 80°C, 10 min						Structural gene has been cloned
licheniformis CIB 6346	30	E	7—9	90	7—10 37°C, 24 h	100% 85°C, 60 min (+Ca, Na) 100% 15°C, 15 min (−Ca)		62,650 (1)				ATCC number 9789
licheniformis CIB 6816	30	E				100% 85°C, 60 min (+Ca, Na)						
licheniformis CIB 7224	30	E				100% 85°C, 60 min (+Ca, Na)						
licheniformis CIB 8061	30	E				100% 85°C, 60 min (+Ca, Na)						
licheniformis CIB 8537	30	E				100% 85°C, 60 min (+Ca, Na)						
licheniformis CIB 8549	30	E				100% 85°C, 60 min (+Ca, Na)						
licheniformis CIB 8874	30	E				100% 85°C, 60 min (+Ca, Na)						
licheniformis CIB 9668	30	E				54% 85°C, 60 min (+Ca, Na)						
licheniformis CTC 8233	30	E				98% 85°C, 60 min (+Ca, Na)						
licheniformis P01		E		100		100% 80°C, 10 min						Structural gene has been cloned
cillus macerans TCC 8514	40	I	6.2—6.4				3.33 2.62 m*M* (10) 2.65 m*M* (11)	139,300 (5)				Cyclodextrinase
cillus natto IAM 212	30	E	6.1		5—9 4°C, 20 h	50% 57°C, 5 min 6% 65°C, 5 min	1.92	34,000 (4)				Saccharifying amylase; structural gene has been cloned

TABLE 3 (continued)
Properties of α-Amylases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp optimum (°C)	pH stability[b]	Thermostability	K_m (mg/ml)[c]	Mol wt[d]	Isoelectric point	Effect of Ca^{2+}[e]	Inhibitors	Miscellaneous
cillus stearo-hermophilus		I					0.84	39,000 (4)		+	Cu^{2+}, Hg^{2+}, Pb^{2+},	
		E						47,000 (4)		+	Zn^{2+}, EDTA, PCMB	
stearothermo-hilus 503-4	55	E	4.6—5.1	55—70		100% 70°C, 24 h 71% 85°C, 20 h	1.0	15,600 (3) 15,688 (5)	4.82	+	EDTA	Dimeric enzyme; results not reproducible
stearothermo-hilus 510-4	55	E	5.0—6.0	70—80		67% 90°C, 10 min 35% 90°C, 30 min						
stearothermo-hilus 1503-4	55	E	5.4—6.1		6.5—9.0 25°C, 1 h	80% 70°C, 45 min (+Ca) 55% 60°C, 45 min (−Ca)		53,000 (3)	9.3	+	Guanidine hydrochloride	Resistant against proteases; BSA and substrate stabilizes; EGTA inactivates at elevated temperatures
stearothermo-hilus 1518	35 55	E E	6.5—8.0 6.5—8.0	45—55 60—70	5.0—9.5 5.0—9.5	10% 90°C, 60 min 90% 90°C, 60 min						KCl and NaCl also reactivates dialyzed preparations
stearothermo-hilus ATCC 954	60	E			5.75—7.0 60°C, 90 h	75% 90°C, 3 h						
stearothermo-hilus ATCC 2980	58	E	5.5	80	5.5—8 70°C, 2 h 7 70°C, 4 h	95% 70°C, 2 h 50% 87°C, 60 min		59,000 (1,2)	8,8	+		Structural gene has been cloned
stearothermo-hilus ATCC 1199			5.5	65		100% 65°C, 3 h 20% 80°C, 2 h		58,000 (1)				*Structural gene has* been cloned in several staphylococcal strains; properties from cloned produce
stearothermo-hilus BS-1	37	E		70		2% 80°C, 30 min (+Ca)	0.05 (37°C) 0.39 (65°C)			+		

	55	E				17% 80°C, 30 min (+Ca)	0.03 (37°C) 0.04 (65°C)			+		
stearothermophilus CU21						95% 70°C, 60 min 60% 80°C, 60 min		58,779 (2) 53,000 (1)	6.9;8.8			Structural gene ha been cloned; pro erties from the cloned product
stearothermophilus Donk BS-1	43	E	4.5—6.0	70	6—12 25°C, 60 min 8—12 25°C, 20 h	68% 80°C, 10 min	0.77	44,000 (3) 41,000 (4)		+		Mol wt from clon gene 61,000; san as *B. stearothermophilus* DY-5
	55	E	4.5—6.5 (37°C) 5.0—6.0 (60°C)	70	6—11 26°C, 30 min	40% 75°C, 30 min	0.77	46,000 (3) 44,000 (4)				
					8—11 26°C, 20 h	20% 75°C, 30 min						
	55	E	4.5—6.0	70		48% 80°C, 10 min	0.76	43,000 (3) 41,000 (4)				
stearothermophilus KP 1064	60	E	5.8	55	6—9.5 26°C, 15 h 6.5—7.5 50°C, 15 h	100% 65°C, 10 min 15% 75°C, 10 min	50 7.6 (1) 33 (2) 260 (13)	115,000 (4) 59,000 (1)	4.4		Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, PCMB, 5,5′-dithio*bis*(2-nitrobenzoate), L-his, Tris	2 Identical subuni produces maltos and maltotriose; degrades also pullulan
acillus subtilis			5.2—6.4	55	5.0—11.0 20°C, 2 h 6.5—11.0 20°C, 20 h					+		Trademark Biolase,® producer Kalle & Co., FR
subtilis	E		5.8	63 79		80% 60°C, 1.5 d (+Ca) 65% 60°C, 6 d				+		Two forms, proba bly isoenzymes. Produces maltotetraose
subtilis	37	E	6.0—6.5	55		15% 60°C, 5 min 0% 60°C, 15 min						
subtilis	37	E	6.5	55		12% 60°C, 5 min 0% 60°C, 15 min						
subtilis	37	E	6.0	55		9% 60°C, 5 min 0% 60°C, 15 min						
subtilis	37	E	5.5	55		10% 60°C, 5 min 0% 60°C, 15 min						
subtilis	37	E	6.0	55		0% 60°C, 5 min						
subtilis	37	E	5.5	55		0% 60°C, 5 min						
subtilis	37	E	6.5	55		0% 60°C, 5 min						

TABLE 3 (continued)
Properties of α-Amylases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp optimum (°C)	pH stability[b]	Thermostability	K_m (mg/ml)[c]	Mol wt[d]	Isoelectric point	Effect of Ca^{2+}[e]	Inhibitors	Miscellaneous
subtilis	37	E	6.5	55		5% 60°C, 5 min 0% 60°C, 15 min						
subtilis			5.5—6.0	70		100% 55°C, 60 min (+Ca)				+		Substrate stabilizes; trademark BAN 240 S®, producer Novo A/S, Denmark
subtilis			4.5—6.0	55—60	4.5—6.0 55°C, 1 h (+Ca)	100% 55°C, 60 min (+Ca)				+		Substrate stabilizes; trademark Amylase 80z®
					5.5—6.0 55°C, 1 h (−Ca)						Producer Rapidase, France	
subtilis			5.5—6.0	55—60		100% 55°C, 60 min (+Ca)				+		Substrate stabilizes; trademark Dexlo 50®; producer Rapidase, France
subtilis			5.5—6.0	55—60		85% 80°C, 60 min (+Ca) 40% 85°C, 60 min (+Ca)				+		Substrate stabilizes; Trademark α-amylase ZF 178®; producer Institut fur Enzymologie, DDR
subtilis			5.3—6.8		4.8—8.5	65% 60°C, 5 min						
subtilis			5.25	56		20% 65°C, 60 min (+Ca) 2% 65%°C, 2 h (+Ca)		48,000 (4)				Trademark Nagase®
subtilis				43—58		0%, 90°C, 6 min						Producer Daiwa Kasei Co., Japan
subtilis 110			5.0	60		50% 65°C, 60 min (+Ca) 5% 65°C, 5 h		28,000 (4)				
subtilis 128	37	E				10% 65°C, 20 min						
subtilis 168	37	E	5.5			30% 65°C, 10 min 16% 85°C, 60 min (+Ca, Na)						Marburg strain

. subtilis 196	37	E	5.5			30% 65°C, 10 min 10% 65°C, 20 min			Mutated from *B. subtilis* 168; produces 3 times mo α-amylase than strain 168
. subtilis 207-SV1	37	E		70		60% 80°C, 60 min			Liquefying enzyme transformed from *B. amylo-liquefaciens*
. subtilis 6160	30	E	6.8		5—9 4°C, 20 h	50% 67°C, 5 min 70% 65°C, 5 min (+Ca)	1.22	55,000 (4)	Saccharifying enzyme
. subtilis G63	30	E	6—7	50	6—8 25°C, 3 h	100% 50°C, 10 min (+Ca) 70% 55°C, 10 min (+Ca)		25,000 (4)	Produces maltotrios
. subtilis M3	30	E				50% 55°C, 13 min (−Ca) 58% 55°C, 90 min (+Ca)		55,000 (4)	Mutated from *B. subtilis* NA64
. subtilis M9	30	E				50% 55°C, 2 min (−Ca) 55% 55°C, 10 min (+Ca)		60,000 (4)	Mutated from *B. subtilis* NA64; Enzyme cannot be purified because o lability
. subtilis M18	30	E				50% 55°C, 12 min (−Ca) 23% 55°C, 90 min (+Ca)		55,000 (4)	Mutated from *B. subtilis* NA64
. subtilis M20	30	E				50% 55°C, 3 min (−Ca) 20% 55°C, 90 min (+Ca)		37,000 (4)	Mutated from *B. subtilis* NA64
subtilis NA20	30	E	6.1		5—9 4°C, 20 h	50% 57°C, 5 min 8% 65°C, 5 min (+Ca)	3.00	42,000 (4)	Transformed from *B. subtilis* 6160; saccharifying enzyme
subtilis NA64	30	E	6.8	60	5—9 4°C, 20 h 6—8 30°C, overnight	50% 63°C, 5 min 0% 80°C, 5 min	2.30	55,000 (1,4)	Transformed from *B. subtilis* 6160; saccharifying enzyme; 3 cell-bound forms
subtilis NRRL 3411		E	6.0	60	5.5—9.5 70°C, 1 h	81% 70°C, 45 min (+Ca, Na)		48,420 (4) 44,900 (3)	Maximal activity deserves Ca^{2+} and Na^{+}; producer Monsanto Co., U.S.; liquefying enzyme

TABLE 3 (continued)
Properties of α-Amylases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp optimum (°C)	pH stability[b]	Thermostability	K_m (mg/ml)[c]	Mol wt[d]	Isoelectric point	Effect of Ca^{2+}[e]	Inhibitors	Miscellaneous
btilis PB	30	E	6—7					93,000 (1)	5.0;5.6			Saccharifying enzyme
btilis R-623 nt P	37	E	4.5				2.0 2.0 (1)	58,000 (1)	5.6			Morphological variant
btilis R-623 nt R	37	E	4.4			90% 60°C, 60 min	3.5 1.7 (1)	57,000 (1)	5.4			Morphological variant
btilis R-623 nt S	37	E	5.0				5.0 0.9 (1)	56,000 (1)	5.1			Morphological variant
btilis W 23	37	E	6.3	65								
btilis YY88	37	E				100% 50°C, 15 min 30% 60°C, 15 min		67,000 (1,4)				Also membrane-bound forms
btilis var. *losacchari-*	37	E	6.8	43	5—9 4°C, 20 h	50% 68°C, 5 min	2.31	46,800 (5)				Saccharifying enzyme; producer Ueda Kagaku Co., Ltd., Japan; degrades cyclodextrins
btilis var. *imus*	37	E	7.0	45		50% 60°C, 15 min						
riodes amylo-us 70	37	E	6.3	43	5.8—7.5 25°C, 24 h	18% 55°C, 90 min	18.2 μmol 18.2 μmol (1,2) 16.7 μmol (4)	92,000 (1)	4.6	+	Hg^{2+}, α-, and β-cyclodextrin, α-D-phenylglucoside	Co^{2+} also activates
idium ricum	37	E	5.3—5.5	46—48								
tyricum	40	E	4.6—6.1	48								
acterium sp.				25 (5% NaCl)		80% 25°C, 6 d 0% 50°C, 6 d						
				40 (15% NaCl)		80% 40°C, 6 d 0% 60°C, 6 d						
			5.6	55 (25% NaCl) 6.8		80% 50°C, 6 d 0% 70°C, 6 d						

acterium bium	37	E	6.4—6.6	55							Does not need Ca^{2+} but NaCl and KCl activate after removal of Na^+
bacillus LEM	37	I	5.0	55							
bacillus LEM	37	I	6.4	40							
bacillus LEM	37	I	5.5	55							
bacillus cel-osus D-39	37	E	7.3	50	6.3—7.9 4°C, 24 h	100% 50°C, 60 min 20% 70°C, 60 min		22,500 (1) 24,000 (4)			
coccus hal-s ATCC 27	30	E	6—7	50—55	6.5—7.5 50°C, 30 min	100% 30°C, 30 min 40% 60°C, 30 min		89,000 (1)		+	EDTA	$CaCl_2$ and NaCl or KCl necessary for activity. Completely inactivated after release of Na^+
coccus vari- subsp.	30	E	6—7	55 (−Ca) 60 (+Ca)	7—9 50°C, 30 min (−Ca) 5.5—9 50°C, 30 min (+Ca)	100% 48°C, 30 min (±Ca) 50% 60°C, 30 min (+Ca)		86,000 (1)		+		
		E	6—7	55 (−Ca) 60 (+Ca)	7—9 50°C, 30 min (−Ca) 5.5—9 50°C, 30 min (+Ca)	100% 48°C, 30 min (±Ca) 50% 60°C, 30 min (+Ca)		60,000 (1)		+		
lomonas MS1		I	5.5	50	4.5—7.5 40°, 30 min	95% 50°C, 30 min 0% 60°C, 30 min		3.3 (1) 13 (2) 1.0 (6) 1.4 (7) 3.5 (10)	96,000 (1)		Ag^+, Cd^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Pb^{2+} EDTA	Cyclodextrinase
lomonas harophila		E	5.25—5.75	40	4.5—8.0 20 h RT		0.6					
ccharophila	30	E	5.4									
ococcus sp.	40	E	5.5—6.5	48						+		
ococcus nus 1091	37	E	7.0 (+Ca) 4.75;8.0 (−Ca)	38						+	EDTA	Cl^- also necessary for activity
sophile V2	65	E	6.0—7.0	70	5.4—10.6 25°C, 24 h 9.2 80°C, 30 min	100% 80°, 2 h 50% 90°, 1 h	14μmol bonds/ml	50,000 (1)	9.2	+		Sr^{2+} and Na^+ also stabilize. Effect of Na^+ and Ca^{2+} additive

TABLE 3 (continued)
Properties of α-Amylases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp optimum (°C)	pH stability[b]	Thermostability	K_m (mg/ml)[c]	Mol wt[d]	Isoelectric point	Effect of Ca^{2+}[e]	Inhibitors	Miscellaneous	
ACTINOMYCETAL													
omyces	40	E	6.0		7.0 30°C, 20 min	100% 60°C, 10 min (+Ca) 60% 70°C, 10 min (+Ca)							26
omyces aureus BM-K		E	4.6—5.3	40	6—8 30°C, 30 min	90% 50°C, 15 min 35% 60°C, 15 min		40,000 (4)		+/−	Ag^+, Cu^{2+}, Hg^{2+}, PCMB, monoiimidoacetic acid, wheat amylase inhibitor		27
omyces cellu- 3313	30	E	6.5		7.0 30°C, 20 min	100% 40°C, 10 min (+Ca) 50% 50°C, 10 min (+Ca)				+			26
omyces diastus 3315	30	E	6.75							+			26
omyces griseus	30	E	6.75		7.0 30°C, 20 min	33% 45°, 10 min (+Ca) 17% 45°, 10 min (−Ca)				+			26
omyces hygroscopicus SF-		E	5—6 6.0	50 63	5—10 40°C, 60(+sub)min 6—9 40°C, 60(−sub) min	100% 55°, 15 min 60% 70°C, *15 min*		48,000 (1)	5.1	+	Ag^+, Hg^{2+}, SDS, amylase inhibitors	Substrate stabilizes	27
omyces limosus 5131	30	E	7	35		100% 45°C, 60 min 65% 50°C, 60 min						EDTA and PCMB does not inhibit; also degrades pullulan	27
omyces mitis 3332	30	E	6.75		7.0 30°C, 10 min	100% 40°C, 10 min (+Ca) 38% 50°C, 10 min (+Ca)				+			26
omyces pre- NA-273		E	6.0—7.0	40	6—8	100% 40°C 0% 60°C							27

oactino- *s* sp. No. 15	55	E	7	80	8 55°C, 24 h	100% 70°C, 30 min 74% 100°C, 30 min		47,800 (4)			Hg^{2+}, Zn^{2+}	Substrate does not stabilize; saccharifying enzyme; Ag^{+}, Co^{2+}, Fe^{2}, Mn^{2+} activate	1
noactino- *esvulgaris* 5	55	E	5.6—7.0	60	5.9 60°C, 80 min (+Ca)	90% 60°C, 16 h (+Ca) 50% 65°C, 16 h (+Ca)						Two isomers, another possibly dimer; Saccharifying enzyme	2
lgaris 42	45	E	5.0—6.0	65		100% 85°C, 60 min (+Ca) 70% 90°C, 60 min (+Ca)		22,500 (1,4)			Hg^{2+}, PCMB		2
lgaris R-47	50	E	5.0	70		100% 60°C, 2 h (+Ca) 60% 60°C, 2 h (−Ca)			5.2	+	Al^{3+}, Cu^{2+}, Hg^{2+}, Fe^{3+}, PCMB, maltotriitol, panitol, isopanitol, amylase inhibitors	Mn^{2+} also stimulates; saccharifying enzyme; cleaves also some 1,6-glucosidic bonds	2
nomospora *ata*	53	E	5.5—6.5	65	7—8 65°C, 30 min	60% 90°C, 5 min (−BSA) 0% 90°C, 5 min (+BSA)	0.39	62,000 (1)				Produces mixture of maltotetraose and -pentaose	1
nomonospora *aris*	55	E	6.0	60	7.0 2 h	100% 35°C, 100 min 35% 60°C, 80 min	1.4%				Cd^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} KCN, EDTA, iodoacetate	BSA stabilizes; tolerates 8 *M* urea	2
FUNGAL													
gillus *nori*			5.0	40	6—7	85% 55°C, 10 min 40% 60°C, 10 min					Ag^{+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, halides	Substrate stabilizes	2
amori ATCC 2	30	E	4.8—5.0	50	3.5—6.5 24 h	85% 40°C, 60 min 40% 60°C, 60 min	1.0	54,000 (1)	4.2	±	Hg^{2+}, Pb^{2+}, maltose		2
gillus batatae		E	5.1				0.53						2
gillus candi- *var.* *olyticus*	30		5.3										2
gillus cheva- NSPRI 105		E	5.5	40		44% 60°C, 15 min 0% 70°C, 15 min	0.19	68,000 (4)		+	EDTA, DNP	Mg^{2+} also stimulates	2
gillus flavus			5.25	50	5—8	95% 55°C, 10 min 70% 60°C, 10 min					Ag^{+}, Cu^{2+}, Hg^{2+}, halides	Substrate stabilizes	2
gillus foeti- ATCC 10254	28	E	5.0	45		100% 35°C, 60 min 7% 55°C, 60 min	2.19 7.65 (1) 2.19 (2)	41,500 (4)				NRRL No. 337	2
gillus *ebergi* hwitz	35		5.5	50		100% 40°C, 15 min 40% 60°C, 15 min		50,000 (4) 3.9					2

TABLE 3 (continued)
Properties of α-Amylases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp optimum (°C)	pH stability[b]	Thermostability	K_m (mg/ml)[c]	Mol wt[d]	Isoelectric point	Effect of Ca^{2+}[e]	Inhibitors	Miscellaneous	Ref.
Aspergillus niger		E E	4.0—5.0 5.0—6.0		2.2—7 37°C, 30 min 5—8.5 37°C, 30	88% 60°C, 15 min 27% 70°C, 15 min 85% 40°C, 15 min 25% 50°C, 15 min		58,000 (3) 61,000 (3)	3.44 3.75	+ +		Trademark Sanactase,● producer Meiji Seika Kaishi Ltd., Japan; 2 forms, acid stable and unstable	290—294
A. niger ATCC 13469	30	E	5	50	4—6 30°C, 24 h	Below 60°C							295
A. niger van Tieghem CFTRI 1105	40	E	5.0;6.0	60	5.2—6 (+Ca) 5.8—7 (−Ca)	100% 65°C, 10 min (+Ca) 15% 65°C, 10 min (−Ca)	3.3	56,230 (1)		+	Ag^+, Al^{3+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+} EDTA	Produces 2 forms; substrate stabilizes; also NaF and $MgSO_4$ stimulate	296—298
Aspergillus oryzae			5.5—5.9		5.5—8.5 20°C, 20 h			52,600 (3)	4.2			Trademark Clarase;● producer Miles Laboratories, U.S.	299
A. oryzae			5.0—5.5	55	4.7—6.0 55°C, 1 h (+Ca) 5.5—6.0 (−Ca)	100% 55°C, 60 min (+Ca)				+		Trademark Fungamyl 800L;● producer Novo Industri A/S, Denmark	241
A. oryzae			5.0	40	6—8	75% 55°C, 10 min 20% 60°C, 10 min					Ag^+, Cu^{2+}, Fe^{3+}, Hg^{2+}, halides	Substrate stabilizes	283
A. oryzae			4.8—6.6	35—37			7.13 m*M* (10) 4.35 m*M* (11) 3.12 m*M* (12)					Cyclodextrinase, Producer Biogal Pharmaceutical Works, Debrecen, Hungary	300
A. oryzae			5.0—5.9		5.8—7.2 10°C, over a year 5.0—8.2 37°C, 30 min	70% 60°C, 90 min (+Ca) 70% 50°C, 30 min (−Ca)	29% 2.4% (3) 4.7 m*M* (10) 10.2 m*M* (11) 2.4 m*M* (12)	53,000 (2)		+	PCMB	Trademark Takadiastase Sankyo,● "Taka-Amylase A"; producer Sankyo Co., Ltd., Japan	128, 301—304

zae 245		E	5—6	30—40			4.16				ATCC number 9376	3
zae var.		E	5.0	50	6.5—9.0 37°C, 1 h 6.5—7.5 50°C, 1 h	100% 60°C, 60 min (+substrate) 100% 30°C, 60 min (−substrate)				Cu^{2+}, Pb^{2+}	Substrate stabilizes; producer Miles Laboratories Inc., U.S.	3
zae Cohn 125.49	27	E	5.5—5.9		7.6—8.8 25°C, 16 h (+Ca) 8.0—8.8 25°C, 16 h (−Ca)							3
zae EI 212	30	E	4.5—5.0	50—55	5.0—11.0 30°C, 1 h	100% 45°C, 60 min	3.35	56,000 (4)	−	Ca^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+}, Mg^{2+}	EDTA removes Ca^{2+} inhibition	1
						30% 60°, 60 min				Zn^{2+}		
omium nophile	45	E	7	45								3
ium oxyspo- Schl.	27		4.0 6.9	25 30								1
ium scirpi o and Fant r.	27		4.5 6.9	40 40							Saccharifying enzyme	1
ium vasinfec- Atk.		I	4.4—5.0; 5.8 7.8—8.0	45—50	3.8—10.0	70% 50°C, 30 min 12% 60°C, 5 min				Cu^{2+}, Mn^{2+}, Zn^{2+}	3 pH otima	1
cola insolens	45	E	6	50								3
cola	45	E	7	50								3
ginosa *anchea pul-* a var. *rea*	45	E	7	50								3
r miehei						50% 50°C, 80 min 0% 71°C, 20 min					Trademark Marzyme,® producer Miles Laboratories Inc., Madison, WI, U.S.	3
r pusillus	45	E	6	50								3
sillus F3	45	E	4.0—4.5	65—70								3
sillus var.						95% 50°C, 80 min 65% 71°C, 20 min					Trademark Emporase,® producer Dairyland Food Laboratories Inc., U.S.	3
omyces sp. C 46889	30	E	4.0	45	4.0—9.0 RT, 24 h	100% 45°C, 10 min (+Ca) 55% 45°C, 10 min (−Ca)		69,000 (4)	+			3
orus ostrei- *is* IH75164	35	E	5.2	35—37								3

TABLE 3 (continued)
Properties of α-Amylases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp optimum (°C)	pH stability[b]	Thermostability	K_m (mg/ml)[c]	Mol wt[d]	Isoelectric point	Effect of Ca^{2+}[e]	Inhibitors	Miscellaneous
ophyllum nune	26	E	5.2	37	7.3 40°C, 30 min	83% 37°, 30 min 81% 40°C, 30 min		46,000 (4)		+	Hg^{2+}, EDTA	
omyces nti	45	E	7	50								
omyces emer- IMI 170757	45	E	5—6	40—45								
noascus ntiacus	45	E	6	45								
noascus aceus	45	E	7	50								
oderma viride 354.44	29	E	5.0—5.5		4—7 2°C	60°C, 10 min						
YEAST												
da japonica 1906	30	E	5—6	55								
nycopsis cap-is 311/1	30	E	4.5	40—50	4.8—7.0 20°C, 96 h							
nycopsis cap-enum CCY -1	29	E	5.6	50	5.6 35°C, 24 h	100% 40°C, 30 min 35% 50°C, 30 min		64,000 (1)				Same as *Candida japonica*
nycopsis fi-era 241	21	E	5—6	30—40			4.16					
yces kono-oae CBS	25	E	5.5	40	4—6.5 40°C, 60 min	below 40°C	2.7 109 (6)	38,000 (4)	7.1	±	Maltose	
yces starkeyi 1807	28		3—4	50		100% 50°C, 30 min 22% 75°C, 30 min						Bound to cell wall
rkeyi CBS	25	E	4.0	70				76,000 (4)				
burtonii in CBS 6141	28		6.2	45		90% 40°C, 30 min 20% 55°C, 30 min					Ba^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+}, Zn^{2+}	Bound to cell wall
polymorpha	28	E	4.0	40		100% 40°C, 30 min				–	Ba^{2+}, Ca^{2+}, Co^{2+},	Also bound to cell

ker CBS 106						30% 50°C, 15 min				Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, EDTA	wall	
aromycopsis igera HUT	28	E	6.0	40—50							Gene has been cloned	1
nniomyces ius	30	E	6.3	40	4—7 40°, 60 min	100% 40° 60 min	4.76	62,000 (1)				3
vius ATCC 4	30	E						52,000 (1)			3 Forms probably precursors; NRCC strain 2509	3
vius UCD -83	30	E	6.3	40	4.5—7.5 30° 24 h	100% 40°C, 30 min 44% 50°C, 30 min	0.36	61,900 (1)			Glycoprotein; ATCC number 4442	3
nniomyces llii 1402	21	E	5.5—6.5	30—40		100% 40°C, 30 min 32% 50°C, 30 min	1.15				ATCC No. 20677	3
tellii 3754	28	E	4.5—7.2	45								3
tellii Cap- ti CBS 2836	28	E	6.0	60		90% 60°C, 30 min 45% 65°C, 30 min		40,000 (1)	–		Ca^{2+} inhibits at great concentrations	3
opsis ingen- Di Menna 4240	28		5.5	50		100% 40°C, 15 min 10% 50°C, 10 min					Bound to cell wall	3

E denotes extracellular and I intracellular enzyme.
RT means room temperature.
Substrate is indicated by number in parentheses; starch is without number; substrates are (1) amylose; (2) amylopectin; (3) glycogen; (4) dextrin; (5) maltose; (6) maltotriose; (7) maltotetraose; (8) maltope
maltohexaose; (10) α-cyclodextrin; (11) β-cyclodextrin; (12) α-cyclodextrin; (13) pullulan.
Method used to measure mol wt is given in parentheses; (1) SDS PAGE; (2) amino acid or DNA sequence; (3) sedimentation analysis; (4) gel filtration; (5) amino acid analysis; and (6) ultrafiltration.
+ Means activation or stabilization; ± no effect; and − inhibition or destabilization.

properties of component C are the same as those of the extracellular enzyme.[248] K_m values in Table 3 are not comparable because substrates and conditions of measurements, as well as ways of indicating values, have been different. It seems that measurements have not always been done under conditions for optimal enzyme activity.

1. pH Optimum and Stability

pH optimum of α-amylases varies from 2.0 to 10.5, indicative of evolutionary adaptability to environmental circumstances. The pH for optimal growth of the producing microbes varies, respectively, from 3 to 10.5. The pH optimum of *B. stearothermophilus* Donk BS-1 α-amylase is reported to be dependent on temperature,[237] and *B. stearothermophilus* 1503-4 enzyme dependent on calcium.[235] *Streptococcus equinus* α-amylase has in the absence of calcium two pH optima, which do not coincide with the one pH obtained in presence of calcium.[266] Amylases of *Bacillus* strains No. 38-2, 135, and 169 have two pH optima.[161] These results may arise from presence of several different amylolytic enzymes. Amylase from alkalophilic *Bacillus* sp. has three pH optima at 4.0, 9.0, and 11.0, which were suggested to be due to the same enzyme.[162] This, however, may not be the case since the endproducts differ at the different pH values.

2. Temperature Optimum and Stability

Temperature optima for activity of enzyme and for growth of the microbe are related (see Section III.C.4.). The lowest temperature optimum is reported to be 25 to 30°C for *F. oxysporum* amylase[194] and the highest, at least 100°C, for *B. licheniformis* enzyme.[101] Optimum growth temperatures vary from 21°C for *Endomycopsis fibuligera*[305] and *S. castellii*[326] to 72°C for *D. thermophilum.*[120] *B. amyloliquefaciens* F, SB, and T α-amylases have two temperature optima, 45 and 65°C.[204] Likewise, the isoenzymes of *B. subtilis* have different temperature optima, 63 and 79°C.[240] Temperature optima of enzymes from the halophilic *M. varians* are calcium dependent,[158] and the one from *Halobacterium* sp. is NaCl dependent.[155] Thermostabilities have not been measured *de facto* in many studies because of too short an incubation period. Many factors can affect thermostability, including purity of preparate, presence of calcium, substrate, and other stabilizers. The stabilizing effect of substrate is of dual origin. Starch contains usually some calcium as an impurity, and it could be thought that when bound to the substrate the conformation of the enzyme is more rigid and stable against denaturing conditions. Starch does not stabilize amylases of *Thermoactinomyces* sp. No. 15[176] or *T. curvata.*[177]

B. acidocaldarius,[167] *B. stearothermophilus* 1503-4,[235] and *Thermomonospora vulgaris*[282] enzymes are stabilized by BSA, but amylase from *T. curvata* is inactivated faster at 90°C in the presence of it.[177] Stabilization in the presence of BSA is probably due to increased concentration of protein, which is advantageous when proteases are present. This is relevant, because it has been shown that proteases can be present even in highly purified crystallized preparations.[332]

3. Molecular Weight

Molecular weights of α-amylases vary from about 10,000 to 139,000 (see Table 3). The lowest value, 10,000, for *B. caldolyticus*[214] is an estimate obtained by ultrafiltration, a technique which is not intended for quantitative molecular weight determination. Molecular weights of microbial α-amylases are usually 50,000 to 60,000, as shown directly by analysis of cloned α-amylase genes and deduced amino acid sequences. Molecular weights determined by standard biochemical techniques are somewhat unreliable because results are markedly dependent on methods. We must consider that the only very accurate molecular weight determinations are those obtained of enzymes for which the amino acid sequence has been determined and/or from sequenced α-amylase genes.

B. amyloliquefaciens amylase contains 1 mol of zinc per 2 mol of enzyme[333] and forms dimers and higher multimers.[212,334] It has been postulated that zinc chelates imidazole groups of histidyl residues and causes aggregation.[335] Enzymes of *B. amyloliquefaciens* N, P, SB, and T have two isoelectric points, but only one after dialysis against EDTA.[206] This was believed to follow from higher isoelectric points of dimers.[206] α-Amylase dimers have been detected also in *B. stearothermophilus*.[175] Isoelectric focusing gives two values for enzymes of *T. vulgaris*.[278] The effect of zinc on these results has not been studied. The molecular weight of *Bacteroides amylophilus* α-amylase is 92,000, but the possibility of dimers was not excluded.[258]

Carbohydrate moieties raise the molecular weights of some α-amylases. Glycoproteins are detected in *A. oryzae*,[336] *B. stearothermophilus*,[230] in *B. subtilis* strains NA64,[30,205] 6160[30] and YY88,[127] and in *S. castellii*.[326] The proportion of carbohydrates in molecular weight of *S. castellii* enzyme is exceptionally high, 56%,[326] when it is about 10% for the other α-amylases. Glycosylation of bacterial proteins is rare. At least in some cases, obtained carbohydrate moieties can be tightly bound oligosaccharides.

4. Inhibitors

Many metal cations, especially heavy metal ions, sulfhydryl group reagents, EDTA, and EGTA inhibit α-amylases (see Table 3). EDTA, which chelates stabilizing Ca^{2+} ions, does not remarkably inhibit enzymes of *B. acidocaldarius*,[199] *B. licheniformis* 584,[224] and *Streptomyces aureofaciens* BM-K.[270] EGTA inhibits *B. stearothermophilus* 1503-4 α-amylase only at temperatures over 50°C.[235]

Calcium, which stabilizes most α-amylases, inhibits enzymes of *A. oryzae* EI 212,[195] *P. polymorpha*,[322] and at high concentrations *S. castellii*.[328] Calcium inactivates *A. oryzae* EI 212 enzyme, but retains activity after EDTA treatment.[195] Almost all α-amylases contain and require Ca, and it is shown to be essential for the folding of the enzyme in *A. oryzae*.[140] It is astonishing to find α-amylases which are inactivated by calcium because these enzymes should have different stabilization mechanisms and probably also different folding.

5. Immunological Reactivity as Indicator of Different α-Amylase Types

Liquefying *B. amyloliquefaciens* α-amylases bind only anti-α-amylase made against liquefying *B. amyloliquefaciens* enzymes, but do not react with antisera raised against saccharifying *B. subtilis* α-amylase and vice versa.[203] Only 2 out of 95 amylase-producing *B. subtilis* strains reacted with antisera induced by liquefying *B. amyloliquefaciens* enzyme,[337] and it is possible that those two strains were, in fact, *B. amyloliquefaciens*. Liquefying amylases of *B. amyloliquefaciens* and *B. licheniformis* precipitate only antisera made against liquefying enzymes.[96,136,249] Saccharifying amylases have affinity only on antibodies made against saccharifying enzymes.[30] It is not known if this kind of recognition is universal for all saccharifying or liquefying α-amylases, but it does seem to be applicable to related bacterial strains. Plasmid pTUB4, which carries the *amyE* gene of *B. subtilis* NA64, encodes a hybrid protein, of which the C-terminus is coded by the vector.[38] This enzyme has a molecular weight different from the parental amylase, but it interacts with antisera as *B. subtilis* α-amylase.[38]

6. Calcium and Stability of α-Amylase

α-Amylase is a metalloenzyme which contains at least one activating and stabilizing Ca^{2+} ion.[333] The amount of bound calcium varies from one to about ten. Crystalline Taka-amylase A contains ten Ca^{2+} ions but only one is tightly bound.[338] Enzymes of *B. subtilis* and *B. stearothermophilus* contain four Ca^{2+} ions.[339] In the *B. amyloliquefaciens* enzyme, which binds relatively tightly four calcium ions, the last one has the greatest effect on stability and conformation.[340] In other systems, usually one calcium is enough to stabilize

the enzyme. The role of calcium may be in tightening the binding between domains of the α-amylase.[341]

One of the conserved regions of α-amylases is suggested to be responsible for calcium binding.[137] Modeling of structures of liquefying *Bacillus* α-amylases indicated that also some other conserved residues are involved in Ca binding.[341] Ca^{2+} is bound outside the active center of the *B. amyloliquefaciens* enzyme,[340] to the SH group of a cysteine in the *B. subtilis* enzyme,[342] and to a carboxyl group in the *Thermophile* V2 α-amylase.[268]

Ca^{2+} can be removed from amylases by dialysis against EDTA or by electrodialysis.[333,343] Secondary Ca^{2+} ions, which do not affect stability, can be freed by quite mild treatments. Loosening of tightly bound Ca^{2+} from *B. subtilis* α-amylase causes a conformational change above, but not below, 15°C.[344] Calcium-depleted *B. caldolyticus* amylase is reported to be stable at 45°C,[345] but in these experiments the substrate contained calcium; thus, the conclusions may not be warranted. Calcium-free enzymes are reactivated by adding Ca^{2+}. *B. subtilis* amylase needs four Ca^{2+} ions to reach full activity,[344] whereas, one is enough for the *A. oryzae*[346] and *B. subtilis* var. *amylosacchariticus* enzymes.[347]

Some studies have been carried out on the ability of other ions to replace calcium. Displacement of calcium in *B. caldolyticus* amylase by strontium was noticed to occur to some extent,[345] but strontium was inhibitory at higher concentrations.[345] In the presence of Sr^{2+}, the enzyme loses its activity at almost the same rate at 70°C as without it.[345] This α-amylase is also active without any cations, and it can bind cations which do not stabilize it, but activate it to some extent, Zn^{2+} and Sr^{2+} to the same extent, but Mg^{2+} only to about 80% as Ca^{2+}.[345]

The tightly bound calcium has been substituted in Taka-amylase A by strontium and to a lesser extent by magnesium in successive crystallizations in the absence of Ca^{2+} and in the presence of excess Sr^{2+} or Mg^{2+}.[348] EDTA-inactivated Taka-amylase A can be reactivated by Sr,$^{2+}$ Mg,$^{2+}$ and Ba^{2+}.[348] These ions also stabilize to some extent enzymes from *B. stearothermophilus*,[235] whereas, no effect was detected on amylase of *Bacillus* sp. AK-2.[197] Ca^{2+} and Mg^{2+} reactivate *B. acidocaldarius* enzyme after dialysis,[166] whereas Ca^{2+} and Sr^{2+} enhance the thermostability of *Thermophile* V2 enzyme.[268]

B. amyloliquefaciens α-amylase can have its Ca^{2+} replaced with rare earth metal ions, with different effects on activity.[350] *B. subtilis* α-amylase could not be activated with lanthanides.[351] Apparently, rare earth metal ions do not compete for its calcium-binding site.[351] The authors suggest that the *B. subtilis* enzyme has two calcium-binding sites, one of which can bind Gd^{3+}, but the calcium-binding site essential for activity and stability can not bind Gd^{3+} or other lanthanides.[351] They show that even atom adsorption spectrophotometrically pure reagents have enough contaminants to give erroneous results.[351]

In the presence of calcium, α-amylases are much more thermostable than without it (see Table 3). Amylase of *H. halobium* is stabilized by Na^{+},[156] and Na^{+} and Ca^{2+} have a cumulative effect on thermostability of *Thermophile* V2 enzyme,[267,268] where they bind to different sites.[268] Enzyme of *B. licheniformis* CUMC 305, which is stabilized by Ca^{2+} and Na^{+}, is reactivated only by Cu^{2+} and Fe^{2+}.[226] It is generally postualted that thermostable enzymes are more resistant to proteases than mesophilic enzymes, e.g., thermostable L-asparaginase and β-galactosidase.[352] This seems to be true, at least to a limited extent, for α-amylases also. Proteolytic degradation does not occur efficiently if calcium is present in α-amylase.[353] Stability with respect to proteases is an important property in consideration of heterospecific protein production from cloned genes since foreign proteins may be readily attacked by proteases produced by the host organism.[354] It is still unclear if calcium can be replaced by other cations. Affinity of Ca^{2+} to α-amylase is much stronger than of other ions. It is so strong that small contaminating amounts of calcium in reagents and glassware can give erroneous results.

Irreversible thermal inactivation of *Bacillus* α-amylases occurs via formation of scram-

bled structures or deamidation of Asn or Gln residues.[355,356] The extra thermostability of the thermophilic *Bacillus* α-amylases is due to additional salt bridges, which reduce the extent of unfolding of the enzyme.[356] The thermostable enzymes are more rigid, and thus their lower tendency to form incorrect structures leads to higher thermotolerance.[357]

7. Structure of α-Amylase

Active site of α-amylase has been studied by chemical modifications, kinetic analyses, and difference spectrophotometric studies. Amylolytic enzymes have subsites which each bind glucose units of substrate and form the active center.[358] Tryptophan has been found to be present in subsites of enzymes produced by *B. amyloliquefaciens*,[359,360] *B. subtilis* var. *amylosacchariticus*,[361-363] *B. subtilis*,[364-366] and *A. oryzae*.[367] Tyrosine has been found in *B. amyloliquefaciens*,[360,368,369] *B. subtilis* var. *amylosacchariticus*,[361] and *B. subtilis*[364,366] amylase subsites. Histidine and carboxy acid groups have been found in the active site of *B. amyloliquefaciens* enzyme.[370,371] These studies provide some information on possible amino acids in the subsites, but do not, of course, determine the complete amino acid constitution of the active sites because reagents used are not absolutely specific for certain amino acids and the microenvironment in the protein can have a significant effect on results.

Taka-amylase A of *A. oryzae* and porcine pancreatic enzyme are thus far the only studied α-amylases to be analyzed by X-ray crystallography.[140,372-374] Three-dimensional structure of liquefying *Bacillus* α-amylases have been modeled.[341] According to proposed subsite binding models, seven glucose units of substrate are bound each by at least two amino acids on the surface of the molecule.[140] Calcium is bound quite near to the active center and seems to stabilize the cleft in the active center.[140]

The catalytic site is formed by two residues which are in Taka-amylase A Asp-206 and Asp-297 and in porcine pancreatic enzyme, respectively, Asp-197 and Asp-300.[374] The substrate is bound by the residues located in the walls of the active site cleft. The residues are quite conserved (Figure 2), and sequence comparison of several predicted secondary structural features imply high conservation of the structure,[375] too. Protein engineering study of the *B. stearothermophilus* α-amylase revealed that Asp-331 is presumably in the catalytic site.[341]

IV. EXOAMYLASES PRODUCING α-TYPE ENDPRODUCTS

Several exoacting amylases have been described in addition to β-amylase, glucoamylase, and α-glucosidase. These enzymes produce short saccharides from the nonreducing end of the substrate. *Pseudomonas stutzeri* enzyme can function both in exo- and endo- fashion.[376] *Aerobacter aerogenes* enzyme is typed as exo-maltohexaohydrolase (1,4-α-D-glucan maltohexaohydrolase EC 3.2.1.98)[377] and *P. stutzeri* enzyme as maltotetraose-forming enzyme (1,4-α-D-glucan maltotetraohydrolase EC 3.2.1.60).[376]

Properties of these enzymes resemble those of α-amylases, i.e., pH optima vary from 5.3 to 7.0, temperature optima from 45 to 62°C, and mol wt from 47,000 to 93,000 (see Table 4). They are stabilized by calcium, as are α-amylases. The properties of the *P. stutzeri* enzyme are according to the report of Sakano et al.,[384] though they have been characterized several times.[376,386] *A. aerogenes* enzyme is found bound to cell walls.[378] In the others microbes the enzyme is found from extracellular fluids. A UV-induced mutant of *A. aerogenes* produces extracellular enzyme.[380] The gene coding for the *B. stearothermophilus* exoacting maltogenic amylase has been cloned into *B. subtilis* and used for industrial production.[383]

The endproducts of amylolysis differ, but all studied so far have α-configuration. Enzyme of *B. stearothermophilus* produces maltose,[383] *Streptomyces griseus* mainly maltotriose,[274] and *Bacillus circulans* F-2 first maltohexaose, which is then split into maltotetraose and

TABLE 4
Properties of Exoacting Enzymes Producing α-Type End Products

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp optimum (°C)	pH stability	Thermostability	K_m (mg/ml)[b]	Mol wt[c]	Isoelectric point	Effect of Ca^{2+}[d]	Inhibitors	Miscellaneous	
acter aero- *s* IFO-3321	30		6.8	60	6.5—9.0 40°, 60 min	100% 40°C, 15 min 0%, 60°C, 15 min		67,000 (1) 54,000 (4)	6.40	+	Cu^{2+}, Hg^{2+}, Mg^{2+}, Sn^{2+}, Zn^{2+}, iodoacet amide	Exo-maltohexaohydrolase; located on membrane	3
rogenes		E	7.0	52	5.0—10.0	90%, 40°C 65%, 50°C		65,000 (1) 48,000 (4)		+		Exo-maltohexaohydrolase; mutated from strain IFO-3321	3
lus circulans	37	E	6.0—6.5	60	6.5—12.0 30°, 60 min 6.5—12.0 0°, 24 h	100%, 40°C, 30 min 23%, 50°C, 30 min		93,000 (1) 54,000 (4)	4.88 4.93	+	Ag^{+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Pb^{2+}, 5′-GMP, 3′-GMP, 2′,3′-cyclic GMP, guanosine, folic acid	Produces maltohexaose as first product and later maltose and maltotetraose	3
lus stearo- *mophilus*			5.3	62		100%, 60°C, 60 min 75%, 70°C, 60 min		70,000 (1)	8.5		Co^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Zn^{2+}	Produces -maltose; gene has been cloned; degrades also pullulan	3
lomonas *eri* NRRL B-	30	E	6.0—6.5	45	6.5—11.0 30°, 60 min	100%, 30°C, 60 min 0%, 55°C, 60 min	1.6 0.4 (1) 1.6 (3) 50 (5) 4.8 (6) 0.7 (9) 91 (10)	57,000 (1) 47,000 (4)	5.3	+	PCMB, ethoxyformic anhydride, amylase inhibitors	2 isoenzymes	3
omyces gri- NA-468			5.6—6.0	45	4—6	100%, 40°C 10%, 50°C					Cu^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}	Produces mainly maltotriose	2

E denotes to extracellular and I to intracellular enzyme.
Substrate is indicated by number in parentheses. Starch is without number. Substrates are (1) amylose, (2) amylopectin; (3) glycogen; (4) dextrin; (5) maltose; (6) maltotriose; (7) maltotetraose; (8) malto
9) maltohexaose; (10) α-cyclodextrin.
Method used to measure molecular weight is given in parentheses; (1) SDS PAGE; (2) amino acid or DNA sequence; (3) sedimentation analysis; (4) gel filtration; (5) amino acid analysis.
+ Means activation or stabilization, ± no effect, and − inhibition or destabilization.

maltose.[381] The enzyme from *P. stutzeri* liberates first maltohexaose and later also maltotetraose.[384]

α-Amylases yield in the first step random-sized polysaccharides, which can be degraded to small oligosaccharides. α-Amylases of *A. oryzae,*[291] *B. stearothermophilus* KP 1064,[239] *B. subtilis* G3,[245] *Streptomyces precox,*[274] and *T. vulgaris*[280] produce mostly maltose. α-Amylases of *B. circulans*[217] and *B. subtilis*[240] liberate predominantly maltotetraose. *B. circulans* G-6 enzyme first produces mainly maltohexaose, which is gradually hydrolyzed to maltose and maltotetraose and then to maltose and glucose.[219] α-Amylases from *B. cereus* NY-14,[216] *B. licheniformis,*[224] and *B. stearothermophilus* ATCC 12980[108] produce mainly maltopentaose.

V. CYCLODEXTRIN FORMING AND BREAKING ENZYMES

A. CYCLODEXTRIN GLYCOSYLTRANSFERASE

Cyclodextrins are rings formed by α-1-4 glucosidically bound glucose units. Sometimes the names Schardinger dextrins and cycloamyloses are used. Rings of 6, 7, and 8 glucose units are called α-, β-, and γ-cyclodextrins, respectively. Cyclodextrins are formed from starch, amylose, and other polysaccharides by the action of cyclodextrin glucanotransferase, also known by the name cyclodextrin glycosyltransferase (1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano)-transferase (cyclizing) EC 2.4.1.19). It is sometimes called according to the first strain found to produce it, i.e., *B. macerans* enzyme. Cyclodextrin glycosyltransferases (CGTases) catalyze degradation of cyclodextrins and the coupling,[387] homologizing, or disproportionation of starch.[387,388] Cyclodextrins are able to form inclusion complexes with a number of both organic and inorganic molecules and so affect properties of included compounds.[389] Thus they have potential commercial importance.[389]

CGTases have been found only from bacterial sources (see Table 5). They are extracellular, except for the enzyme from *B. macerans* ATCC 8514,[227] which is freed to the culture medium on cell lysis.[406] Strains producing CGTase are maintained at 37 to 40°C. The time required for production has been long, up to 2 weeks, but with synthetic media it has been possible to shorten this to 8 to 10 h (see Table 5). Temperature optima of CGTases fall between 45 and 60°C and pH optima between 4.5 and 7. Alkalophilic enzyme has been shown to have maximal activity at pH 9 to 10.5.[389] *Bacillus* sp. have two forms,[391,393] which differ mainly by their pH and temperature optima. The two forms of *B. megaterium* enzyme differ only in isoelectric points;[402] pH and temperature optima of saccharifying and cyclodextrin-forming activities in *B. subtilis* do not coincide.[390] This suggests the presence of several amylolytic activities, although only one band was detected on SDS-PAGE. CGTases are stabilized by calcium ions. Molecular weights are from 67,000 to 145,000. *B. macerans* IAM 1243 enzyme consists of two identical subunits.[400] The sequenced gene codes for a protein having molecular weight of 74,000.[401] *B. macerans* produces mainly α-cyclodextrin;[400] *Bacillus* sp. 1011,[407] alkalophilic *B. circulans,*[393] and *B. circulans* C31[408] produce β-cyclodextrin, whereas *B. subtilis* No. 313 enzyme produces predominantly γ-cyclodextrin.[409]

Genes coding for CGTase have been cloned from *Bacillus* sp. No. 38-2,[407] *Bacillus* sp. 1011,[410] *B. macerans* IAM 1243,[401] *Klebsiella pneumoniae* M5a1,[405] and *B. subtilis* No. 313[409] and have been sequenced from the first four mentioned taxa. These sequenced genes code for proteins having molecular weights of about 63,000 to 75,000 and contain typical *Bacillus* signal peptides of 27 to 30 amino acid residues, which are cleaved during maturation.[394,401,405,410] The sequences have overall amino acid homology of about 30%.[405] Conserved regions are found to be similar, with similar spacing as in α-amylases.[405,410] Also, *E. coli* cells harboring the gene for *B. subtilis* 1011 CGTase gene secrete the enzyme quite efficiently from the cells;[407] 70% of the produced CGTase was found from the medium;[407] *Bacillus* sp. strain No. 38-2 CGTase is found mainly from the periplasmic space of *E.*

TABLE 5
Properties of Cyclodextrin Glycosyltransferases

Strain	T_{growth} (°C)	Growth time	Location[a]	pH optimum	Temp. optimum	pH stability	Thermostability	K_m (mg/ml)[b]	Mol wt[c]	Isoelectric point	Effect of Ca^{2+}[d]	Inhibitors	Miscellaneous	
us sp. HA3-	37	4 d	E	6.5—8.0	60	6—11, 50°C, 30 min	100%, 70°C, 30 min 5%, 80°C, 30 min		68,000 (1)			Co^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}	EDTA, PCMB, and PMSF do not inhibit; ATCC No. 39612	3
us circulans C 21783	37	2 d	E	4.5—4.7	45	6—10, 60°C, 30 min	100%, 65°C, 30 min (+Ca) 10%, 75°C, 30 min (+Ca)	5.88 (10) 0.39 (11) 0.25 (12)	88,000 (1)	5.4	+		Two forms, probably isoenzymes; one is acid stable, and the other acid unstable	3
	37	50 h	E	7	50	6—9, 60°C, 30 min	100%, 65°C, 30 min (+Ca) 10%, 75°C, 30 min (+Ca)	10 (10) 0.83 (11)	88,000 (1)		+			
us macerans	37	15 h	E			6—7, 5°C, 24 h (+Ca)	67%, 68°C, 10 min (+Ca)				+		Maltose stabilizes	3
cerans	38	2 weeks	E	5—6	55		80%, 70°C, 10 min		67,000 (3)	4.5	+	Cyclodextrins, phenylmercuric chloride, Ag^{+}, Zn^{2+}, Hg^{2+}		3
cerans	37	5 d	E	5.4—5.8	60		82%, 40°C, 30 min 18%, 60°C, 30 min	5.7	75,000 (1) 67,000 (3)	5.4			Does not contain multimers	3
cerans		3 d	E	5.6	45	6.0	Below 60°C for 30 min		78,000 (4)	4.5				3
cerans C 8514	40	10 h	I	6.1—6.2				3.33	139,300 (3)					2
cerans IAM	40	48 h	E	6.0	60	5.5—9.5, 25°C, 60 min	90%, 50°C, 15 min (+Ca) 48%, 55°C, 15 min (+Ca)		145,000 (4) 74,000 (2,4)		+		Two subunits; monomer (mol wt 74.000) is inactive form; gene has been cloned	4
cerans IFO	37	70 h		5.5—5.7	55	8.0—10.0, 30°C, 2h	100%, 55°C, 10 min 70%, 65°C, 10 min							4

Bacillus megaterium No. 5	37	70 h	E	5—5.7	55	7.0—10.0, 30°C, 2 h	100%, 55°C, 10 min 20%, 60°C, 10 min			6.07			Two fractions which have different pI values	402, 403
	37	70 h	E	5—5.7	55	7.0—10.0, 30°C, 2 h	100%, 55°C, 10 min 20%, 60°C, 10 min			6.80				
Klebsiella pneumoniae M5AI	37	20 h	E			6.0—7.5 25°, 24 h (+Ca)	100%, 45°, 15 min (+Ca) 13% 50°, 15 min (+Ca)	47.2% (10) 29.3% (11)	69,000 (2)		+	EDTA		404, 405

[a] E denotes to extracellular and I to intracellular enzyme.

[b] Substrate is indicated by number in parentheses; starch is without number; substrates are (1) amylose; (2) amylopectin; (3) glycogen; (4) dextrin; (5) maltose; (6) maltotriose; (7) maltotetraose; (8) maltopentaose; (9) maltohexaose; (10) α-cyclodextrin; (11) β-cyclodextrin.

[c] Method used to measure mol wt is given in parentheses; (1) SDS PAGE; (2) amino acid or DNA sequence; (3) sedimentation analysis; (4) gel filtration.

[d] (+) Activation or stabilization; (±) no effect, and (−) inhibition or destabilization.

coli.[394] The cloned *K. penumoniae* enzyme is secreted both from *E. coli* and *K. pneumoniae*.[405] This is exceptional because *E. coli* does not usually secrete proteins. Production of the *K. pneumoniae* enzyme is under control of both induction and catabolite repression similar to that in *E. coli*.[405]

B. CYCLODEXTRIN-DEGRADING ENZYMES

Circular cyclodextrins do not have nonreducing ends; thus, exoacting enzymes cannot degrade them. Only a few enzymes in addition to CGTases can open cyclodextrin rings. Cyclodextrin-degrading enzymes are mostly α-amylases (see Table 3), which are produced in *A. oryzae*,[300,411] *B. subtilis*,[249] and *B. subtilis* var. *amylosacchariticus*.[257] α-Amylases of *B. coagulans*,[220] *B. macerans*,[228] *Pseudomonas* Msl,[263] and glucoamylase of *Flavobacterium* sp.[412] hydrolyze cyclodextrins faster than starch. Maltose-forming enzymes of *Bacillus alcalophilus* subsp. *halodurans* can degrade β-cyclodextrin;[413] and exoamylase of *B. stearothermophilus* hydrolyses also cyclodextrins.[383]

VI. β-AMYLASE

β-Amylase (1,4-α-D-glucan maltohydrolase, EC 3.2.1.2) occurs commonly in plants, but reports of its occurrence in microbes are rare. β-Amylase hydrolyzes the penultimate 1,4-bond at the nonreducing chain ends of the substrate, causing inversion of the anomeric configuration of the liberated maltose to β, and cannot bypass α-1,6-glucosidic bonds of branched substrate.[414-416] The undegraded part of the substrate is called β-limit dextrin. Food and beverage industries employ β-amylase to convert starch into maltose solutions.

β-Amylase has been found in the strains mentioned in Table 6 and also in *Streptomyces* spp.,[417] *Bacillus polymyxa* ATCC 8523,[430] *B. megaterium* strains S218,[431] AJ 3355, and AJ 3356,[432] and *A. oryzae* EI 212.[195] Screening of microbial β-amylases has been difficult because the presence of other amylolytic activities impedes finding β-amylases.[415,417,433] Screening has been facilitated by use of α-amylase inhibitors.[423,434]

B. cereus BQ10 was mutated with UV to get strain BQ10-S1,[435] which produces about 25 times more β-amylase than the parent strain and without induction by starch.[435] NTG-treatment of this sporulating strain gave rise to asporogenous rifampin-resistant mutants,[436,437] and futher treatment with NTG increased production by 5.5 times.[438] The physicochemical properties of β-amylase from all these strains are equal.[437,438]

The gene coding for *B. polymyxa* β-amylase has been cloned into a *B. subtilis* strain having almost negligible α-amylase activity.[439] The cloned fragment does not contain the whole gene; thus, the produced active enzyme is a hybrid which contains plasmid vector encoded material.[440] Also, the other cloned *B. polymxa* β-amylase is a hybrid.[425] Thus far, the only entire sequence of microbial β-amylase is from *B. circulans*.[420] The cloned genes contain typical *Bacillus* signal sequences. Sequences for *B. polymyxa* and *B. circulans* are 81% homologous at the amino acid level.[420]

A. PHYSICOCHEMICAL PROPERTIES

β-Amylases are extracellular enzymes. Srivastava et al.[197,230] have reported the existence of intracellular β-amylases, but confirmation of this may need more study since they are the only β-amylases which are inactivated by EDTA and activated by calcium ions, properties which are typical for α-amylases. β-Amylases are produced mainly by mesophilic organisms (see Table 6). *Thermoactinomyces* sp. No. 2[429] and *Clostridium thermosulfurogenes*[426] are the only known thermophilic producers and the only producers of thermostable microbial β-amylases. The *C. thermosulfurogenes* enzyme is stabilized markedly by starch, so that it maintains total activity at 80°C for 2 h in the presence of 5% starch. In the absence of starch, the enzyme is completely inactivated by this treatment.[426] Other stabilizers are not known,

TABLE 6
Properties of β-Amylases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp. optimum (°C)	pH stability	Thermostability	K_m (mg/ml)[b]	Mol wt[c]	Isoelectric point	Inhibitors	Miscellaneous
llus sp. AK-2	50	I	6.5	65			1.13			Ag^+, Cu^{2+}, Fe^{3+}, Hg^{2+}, K^+, Pb^{2+}, EDTA, PCMB	Ca^{2+} activates
llus cereus BQ 10	30	E	6.0—7.0	45—55				160,000 (4)			3 Isoenzymes
		E	6.0—7.0	55				80,000 (4)			
ereus var. *mycoides*	30	E	7	50	5—10, 30°C, 3 h	97% 50°C, 10 min 10% 60°C, 10 min		35,000 (4)		Ag^+, Cu^{2+}, Fe^{2+}, Hg^{2+}, PCMB	Calcium does not have any effect
llus circulans IB 11033	37	E		50		100% 42°C, 60 min 34% 60°C, 60 min					Gene has been cloned
egaterium sensu cto NCIB 7581	30	E	6.5—7.0	50	5.0—9.0					PCMB	ATCC No. 15374
egaterium No. 32	30	E	6.5		5.0—7.5, 40°C, 2 h	100% 55°C, 15 min 0% 65°C, 15 min		67,000 (1)	9.1	PCMB	Possibly *Bacillus carotarum*
llus polymyxa IB 8158	30	E	6.8	37	6.8 37°C, 1 h 6.4—7.2, 20°C, 1 h	90% 37°C, 6 d 20% 45°C, 60 min				Cu^{2+}, Fe^{3+}, PCMB, cyclodextrins, erythritol	
olymyxa No. 72	30	E	7.0—8.0	45	4.0—9.0, 37°C, 5 h	100% 50°C, 30 min 15% 55°C, 30 min		44,000 (4) 67,000 (1)	8.35	PCMB	2 Isoenzymes
		E	7.0—8.0	45	4.0—9.0, 37°C, 5 h	100% 50°C, 30 min 15% 37°C, 5 h		44,000	8.59		
llus stearo-rmophilus		E I					1.11 1.11	58,000 (4) 67,000 (4)		EDTA, PCMB	Ca^{2+} also activates EDTA-treated preparate
llus subtilis IMD	30	E	4.8—6.8								
tridium thermosul-ogenes 48	60	E	5.5—6.0	75	3.5—6.5, 60°C, 60 min	100% 70°C, 60 min 83% 75°C, 60 min				Cu^{2+}, Hg^{2+}, PCMB	ATCC No. 33743, DSM No. 2229; starch stabilizes. Tolerates ethanol
domonas sp. BQ06	30	E	6.5—7.4	45—55	6.5—8.0, 30°C, 24 h	100% 45°C, 15 min 2% 60°C, 15 min		37,000 (4)			
otomyces sp. No.	30	E	6.7	40							
moactinomyces sp . 2	55	E	7	60		70% 70°C, 30 min 29% 100°C, 30 min		31,600 (4)		Hg^{2+}	Mn^{2+} and Fe^{2+} stimulate

[a] E denotes extracellular and I intracellular enzyme.
[b] Substrate is indicated by number in parentheses; starch is without number.
[c] Method used to measure mol wt is given in parentheses; (1) SDS PAGE; (2) amino acid or DNA sequence; (3) sedimentation analysis; (4) gel filtration.

except Ca^{2+}, for strains studied by Srivastava et al.,[197,230] and Mn^{2+} and Fe^{2+} for enzyme of *Thermoactinomyces* sp. No 2.[429]

Reported molecular weights of β-amylases vary from 31,600 to 160,000. The highest value may arise from dimer formation because another fraction having a molecular weight of 80,000 was present in chromatographically purified preparation.[418] β-Amylase seems to form several enzymatically active fragments. The hybrid β-amylases having NH_2-terminal domain of *B. polymyxa* genes produce two or more active fragments,[425,439] which arise presumably from action of proteolytic enzymes.[425] Also, *B. circulans* β-amylase appears as two fragments.[420] Cysteine residues seem to be essential for activity or for active conformation of the β-amylases because PCMB is an efficient inhibitor for most of them. Activity of PCMB inactivated *B. megaterium*,[415] and *B. polymyxa*[423] enzymes can be restored by addition of excess cysteine. Heavy metal ions decrease activity significantly. Cyclodextrins inhibit the enzyme of *B. polymyxa* NCIB 8158,[422] but not the enzyme of *C. thermosulfurogenes*.[427] The *B. polymyxa* ATCC 8523 enzyme has been reported to degrade cyclodextrins,[430] but this could be due to the presence of other enzymes as impurities. Cyclodextrins do not have ends from which β-amylase could proceed.

B. alcalophilus subsp. *halodurans* ATCC 27577 (NRRL B-3881) produces an endoenzyme that resembles in many respects β-amylase.[197,413,441] Further studies are necessary to characterize the precise nature of this enzyme, which produces mainly β-maltose on reaction with starch.[413] The microbe grows at 55°C and secretes enzyme, which has optimum pH and temperature at 9.2 and 50°C, respectively.[413] Three isoenzymes are present in culture medium, and they are capable of hydrolyzing β-cyclodextrin.[413]

VII. ISOAMYLASE

Isoamylase hydrolyzes α-1,6-glucosidic linkages of amylopectin, glycogen, various branched dextrins, and oligosaccharides. It cannot degrade 1,6-linkages of pullulan.[442] Isoamylase does not hydrolyze all 1,6-linkages of β-limit dextrins,[443] probably because of its low affinity towards the shortened side chains. *Pseudomomas amyloderamosa* isoamylase requires at least three glucose residues in branched chains to be capable of hydrolysis.[442] Harada[10] has suggested that isoamylase hydrolyses in an exofashion proceeding from the nonreducing end. Isoamylases are used to debranch starch in production of glucose and maltose.

Isoamylase (glycogen 6-glucanohydrolase, EC 3.2.1.68) has been found from only a few microbial strains (see Table 7). *E. coli* enzyme, which hydrolyzes amylopectin completely,[444] is presented here because its other properties are similar to those of other isoamylases. It is possible that 1,4-linkages were degraded by other activities present. Detailed data are available only for *P. amyloderamosa* SB15 enzyme, whose coding gene has been cloned and sequenced.[445] The *P. amyloderamosa* SB-15 isoamylase has a signal peptide of 26 amino acid residues.[445] The sequence is homologous to α-amylases and CGTases in the three reported regions and has significant homology with carboxy terminus of pullulanase.[445] It is suggested tht the carboxy terminal similarities are involved in cleavage of the 1,6-linkages.[445] Mutant MS1 of *P. amyloderamosa* produces isoamylase constitutively,[446] and the NTG-strain 1168 produces over 500-fold more than the wild type.[10]

A. PROPERTIES OF ISOAMYLASES

B. amyloliquefaciens ATCC 23350 produces extra- and intracellular isoamylases.[447] *E. coli* has intracellular enzyme[444] and *S. cerevisiae* membrane-bound protein,[448] whereas the other known isoamylases are extracellular. All the known microbial isoamylases are thermolabile enzymes which are produced at low temperatures (see Table 7). They have no known stabilizers. pH optima and stabilities of isoamylases occur in the acidic range.

TABLE 7
Properties of Isoamylases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp. optimum	pH stability[b]	Thermostability	K_m (mg/ml)[c]	Mol wt[d]	Isoelectric point	Inhibitors	Miscellaneous	
phaga sp.			5.5	40				120,000 (3)	5.0—5.5		Rather thermolabile	
erichia coli JC 3	32	I	5.6—6.4	45—50			4.5			Cu^{2+}, Hg^{2+}, NH_4^+, Tris, PCMB, iodoacetate	Strain KLF 41; EDTA does not inhibit	
obacterium sp.	28	E	6.3	40	6—9 RT, overnight			121,000 (4)		Ag^+, Co^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}		
nyces kononen-e IGC 4051	25	E	5.6	30			9	65,000 (4)	4.7—4.8		CBC strain 2514; β-Cyclodextrin does not inhibit	
domonas lodera-a SB-15	32	E	3.0—4.0	\$2	3.0—6.0 30°C, 12 h 2.5—7.5 4°C, 24 h	100% 40°C, 10 min 5% 60°C, 10 min	0.1 (2.3) 2 (13)	86,000 (1) 88,000 (4) 80,807 (2)	4.4	Ag^+, Cu^{2+}, Hg^{2+}, PCMB, *N*-bromosuccinimide, 2-hydroxy-5-nitrobenzyl-bromide, I_2, iodoacetate, phenylmercuriacetate, 2,4-dinotrofluorobenzene, maltotriose, and -tetraose, amylose	Gene has been cloned and sequenced	44
haromyces evisiae		I	6.0	25						Hg^{2+}, Mo^{2+}, sodium-borate, glucono-lactone	Membrane-bound enzyme	

E denotes extracellular and I intracellular enzyme.
RT means room temp.
Substrate is indicated by number in parentheses; starch is without number; substrates are (1) amylose; (2) amylopectin; (3) glycogen; (4) dextrin; (5) maltose; (6) maltotriose; (7) maltotetraose; (8) maltope
maltohexaose; (10) α-cyclodextrin; (11) β-cyclodextrin; (12) γ-cyclodextrin; (13) pullulan.
Method used to measure mol wt is given in parentheses; (1) SDS PAGE; (2) amino acid or DNA sequence; (3) sedimentation analysis; (4) gel filtration; (5) amino acid analysis.

Typically, the molecular weight is higher than that for α- or β-amylases, ranging from 65,000 to 121,000. *P. amyloderamosa* isoamylase consists of a single chain[455] having a molecular weight of 81,000.[445] Heavy metal ions inhibit isoamylases, but SH reagents decrease activity only slightly or not at all.[450,454] Enzyme of *P. amyloderamosa* SB15 is reactivated from heavy metal ion inactivation by dialysis or EDTA treatment.[454] Cyclodextrins do not inhibit enzymes from *Cytophaga*[456] and *Lipomyces kononenkoae*.[451] 1,4-Glucosidic bonds seem to inhibit isoamylase, e.g., amylose, maltotriose, and -tetraose inhibit the enzyme of *P. amyloderamosa* significantly.[454]

VIII. PULLULAN-DEGRADING ENZYMES

Pullulan is a linear α-glucan consisting of maltotriose units joined by α-1,6 glucosidic linkages. It is produced in *Aureobasidium pullulans* as long chains composed of about 480 maltotriose units. Pullulan cannot be degraded by α- or β-amylases. Isoamylase, which splits the 1,6-linkages of amylopectin, cannot hydrolyze pullulan. Pullulan-degrading activities have been screened from several microbes,[189,457-460] but only very few positive strains have been found. Some *Bacilli*[461] and yeasts[462] could degrade it. However, in yeasts glucoamylases presumably were responsible for the degradation. Pullulan-degrading activity of *S. limosus*[273] arises probably from the presence of true pullulanase.

Four different types of enzymes hydrolyze pullulan and produce different endproducts (see Figure 3). Many glucoamylases hydrolyze pullulan by liberating glucose units from the nonreducing end. They are described in Section IX. Pullulanase yields maltotriose by hydrolyzing 1,6-linkages. Isopullulanase cleaves the first α-1,4 linkage following the 1,6-bond.[463] α-Amylases from *B. stearothermophilus* KP 1064[239] and *T. vulgaris* strains 42[279] and R-47[280] cut the 1,4-glucosidic bond preceding 1,6-linkage and liberate panose. *B. stearothermophilus* TRS40 enzymes liberate panose from pullulan, but hydrolyze only small amounts of starch.[464]

A. PULLULANASE

Pullulanase (α-dextrin 6-glucanohydrolase EC 3.2.1.41) hydrolyzes 1,6-linkages of pullulan and other branched oligosaccharides. Pullulanases have been found from very few microbes (see Table 8). *A. aerogenes*, in which the enzyme was first found,[474] is now classified as *K. pneumoniae* or *Enterobacter aerogenes*. *K. aerogenes* ATCC 21073 was previously typed as *Escherichia intermediae* ATCC 9750. *B. cereus* BQ10 produces pullulanase in addition to isoamylase.[433] Presence of pullulanase in *E. coli* is uncertain, though it is hypothesized that pullulanase is important in its carbohydrate metabolism.[484] Pullulanase has not been found from *E. coli* strains studied.[459,485] *E. coli* strains used as hosts for cloned *pul* genes do not produce their own pullulanase. Commercial applications are similar to those for isoamylases.

1. Cloning of Pullulanase Genes

The pullulanase gene from *K. pneumoniae* W70 was introduced into the chromosome of *E. coli* and *K. pneumoniae*.[486] *E. coli* produces pullulanase as intracellular protein in lesser amounts than the donor,[486] whereas *K. pneumoniae* with cloned *pul* gene produces 2 to 3 times more extracellular enzyme than the donor.[486] When present in a plasmid, the gene induced higher production both in *E. coli* and *K. pneumoniae*.[486] In *K. pneumoniae* the level of production is increased 20- to 40-fold in comparison to the donor.[486] The sequence shows a putative signal sequence of 19 amino acids.[487]

The *K. pneumoniae* ATCC 15050 pullulanase gene has been cloned in *E. coli* in plasmid and into the chromosome.[488] It is controlled in *E. coli* by *malT* promoter and distributed both in the inner and the outer membranes,[488] whereas when cloned in *K. pneumoniae* it is

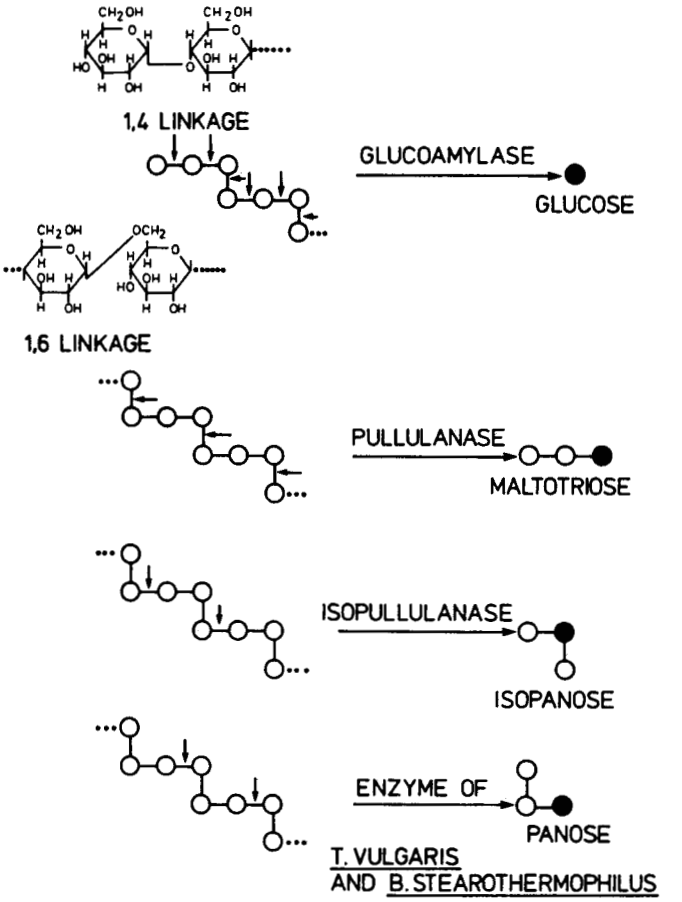

FIGURE 3. Action patterns of pullulan-degrading enzymes. The balls represent glucose units, the horizontal lines 1,4-linkages, and the vertical lines 1,6-linkages.

exported to the outer membrane and released into medium.[488] A single copy of the gene in *E. coli* gave rise to 10 to 20% of the activity of the donor, whereas with a high copy number vector production was about 2 times of that in the donor.[488]

To secrete pullulanase from *E. coli*, a large 18.8-kb fragment containing the surroundings of the *K. pneumoniae* UNF5023 *pulA* gene also has to be cloned.[489] The cloned fragment exhibited maltose-inducible secretion, and the secretion genes are suggested to be part of the maltose regulon.[489] However, transposon mutagenesis revealed that the secretion genes 3′ to the *pulA* may not be regulated by maltose.[489] It seems to be possible to introduce secretion mechanism from one Gram-negative strain to another and possibly use it for specific secretion, e.g., of heterologous proteins. Genes for two thermostable pullulanases have been cloned from *Thermoanaerobacterium brockii* into *E. coli* and *B. subtilis*[482] and from *Thermus* sp. AMD-33 into *E. coli*.[483] Both these genes are cloned in smaller fragments than from *K. pneumoniae* UNF5023, and neither is secreted from *E. coli*.

2. Properties of Pullulanases

Pullulanase appears as a cell-bound, intracellular, and extracellular enzyme. Properties of the intra- and extracellular enzymes in *K. pneumoniae* ATCC 15050 are similar,[476] but the intracellular pullulanase of *K. pneumoniae* No. 105 has a higher molecular weight.[472]

TABLE 8
Properties of Pullulanases

Strain	T_{growth} (°C)	Location	pH optimum	Temp. optimum	pH stability	Thermostability	K_m (mg/ml)[b]	Mol wt[c]	Isoelectric point	Inhibitors	Miscellaneous	Ref.
Bacillus sp. No. 202-1	37	E	8.5—9.0	55	6.5—11 4°C, 24 h	100% 40°C, 15 min 5% 60°C, 15 min		92,000 (4)	2.5	Hg^{2+}, Zn^{2+}	Alkalophilic producer; EDTA and PCMB do not inhibit	465
Bacillus acidopullulyticus			5.0	60	5.0 60°C, 3 d 4.1—5.5 4°C, 3 d			100,000 (1) 101,200 (5)	5.0	Ag^{+}, Cu^{2+}, Co^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, PCMB, α-cyclodextrin	Trademark Promozyme®, producer Novo Industri A/S, Denmark	466
B. acidopullulyticus NCIB 11647		E	5.2				0.37 (3) 0.028 (13)	100,000 (1) 101,000 (5)	5.0	PMB	β-Mercaproethanol reactivates	467
B. acidopullulyticus NCIB 11777		E	4.9				0.30 (3) 0.019 (13)	90,000 (1) 87,000 (5)	5.4			467
Bacillus cereus var. *mycoides*	30	E	6—6.5	50	6—9 30°C, 3 h	95% 40°C, 10 min 50% 50°C, 10 min	0.055%	110,000 (4)		Ag^{+}, Fe^{2+}, Hg^{2+}, PCMB	Ca^{2+} stabilizes	416,
Bacillus macerans NCIB 9368	30	E		50—55		100% 45°C, 10 min 38% 55°C, 10 min					Temp optimum in the presence of Na^{+} at 55°C	468
Bacillus subtilis TU	30	E	7.0—7.5	60				450,000 (4)			Ca^{2+} stabilizes	469
Clostridium thermohydrosulfuricum 39E	65	I	5.5—6.0	85	4.5—5.5 60°C, 60 min	100% 75°C, 60 min 15% 85°C, 60 min	0.33				Also membrane-bound form; substrate stabilizes; tolerates 10% (v/v) ethanol	470
Klebsiella pneumoniae No. 105	30	I	5.5—6.0	50	5.5—13 40°C, 60 min	100% 40°C, 30 min 25% 50°C, 30 min		90,000 (3) 80,000 (4)	3.88 4.46 7.6	Al^{3+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, SDS, EDTA, PCMB	Previously *Aerobacter aerogenes*; Ca^{2+} stimulates	471-
	30	E	6.5	50	5—11.5 40°C, 60 min	90% 50°C, 30 min 15% 60°C, 30 min		66,000 (1) 58,000 (3) 80,000 (4)	3.72 4.53 7.7			
K. pneumoniae ATCC 15050	30		5.0	47.5	4.5—8.0 30°, 20 h 5.0—7.0 40°, 20 h	50% 100°C, 5 min 30% 100°C, 10 min		143,000 (5) 145,000 (3)		Cyclodextrins	Identical extracellular and cell-bound forms. Previously *Aerobacter aerogenes*	456, 47

K. pneumoniae ATCC 21073	30	E	6.0	47				Previously *Escherichia intermedia* ATCC 9750	455, 479
Streptococcus mitis FW 251	39	I	5.4—5.8	30					480
Streptomyces sp. No. 28	30		5.0	60	5.5—7.5 40°C, 60 min	100% 35°C, 60 min 10% 55°C, 60 min			458
Streptomyces sp. No. 280	40		5.0—6.0 (+Ca) 6.0 (−Ca)	50(+Ca) 35(+Na, K or NH_4)		65% 50°C, 60 min (+Ca) 15% 60°C, 60 min (+Ca)	Ba^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}	Ca^{2+}, Mn^{2+} and Zn^{2+} stabilize; produces 10 times more than *Streptomyces* sp. No. 28	481
Thermoanaerobium brockii ATCC 33075	56	E	5.0			50% 70°C, 241 min		Gene has been cloned; three forms mol wt 70,000—100,000 due to proteolysis	482
Thermus sp. strain AMD-33		E	5.5—5.7	70				Gene has been cloned	483

otes extracellular and I intracellular enzyme.
rate is indicated by number in parentheses; starch is without number; substrates are (1) amylose; (2) amylopectin; (3) glycogen; (4) dextrin; (5) maltose; (6) maltotriose; (7) maltotetraose; (8) maltopentaose hexaose; (10) α-cyclodextrin; (11) β-cyclodextrin; (12) γ-cyclodextrin; (13) pullulan.
od used to measure mol wt is given in parentheses; (1) SDS PAGE; (2) amino acid or DNA sequence; (3) sedimentation analysis; (4) gel filtration; (5) amino acid analysis.

It is possible that the intracellular enzyme is a precursor which is cleaved during maturation since *K. pneumoniae* ATCC 15050 enzyme has a proposed signal peptide of 19 amino acids.[490] Pullunase production is induced in *K. pneumoniae*, and its secretion may be controlled by a small molecular weight inductive effector.[491]

Clostridium thermohydrosulfuricum, *T. brockii*, and *Thermus* sp. AMD-33 are the only known thermophilic, pullulanase-producing organisms (see Table 8), and their enzymes are highly thermostable.[470,482,483] Substrate stabilizes markedly the *C. thermohydrosulfuricum* enzyme so that in 5% starch solution, it retains total activity at 85°C for at least 1 h.[470] Pullulanase from *Bacillus acidopullulyticus* is also rather thermostable.[466] Ca^{2+} stimulates *K. pneumoniae* No. 105 enzymes,[473] and enzyme from *Bacillus cereus* var. *mycoides*.[416] The properties of *Streptomyces* sp. No. 280 pullulanase differ by the presence or absence of calcium.[481] *B. macerans* NCIB 9368 enzyme has a higher temperature optimum in the presence on Na^+.[468] pH optima of pullulanases are in the acidic range, except for the enzyme from alkalophilic *Bacillus* sp. No. 202-1 (see Table 8).

Molecular weights vary from 80,000 to 145,000. Molecular weight of the cloned and sequenced *K. aerogenes* KW70 pullulanase is 117,000, and the enzyme is monomeric.[487] Some strains have multiple forms of the enzyme, which, in the case of cloned *T. brockii*, pullulanases are proposed to be proteolytic products.[482] The molecular weight of *B. subtilis* TU pullulanase-amylase complex is exceptionally high, 450,000,[469] and can be due to aggregation of one or more amylolytic activities since the enzyme has different optimal conditions for pullulan and starch hydrolysis.[469] Pullulanases are inhibited by heavy metal ions and cyclodextrins. The enzyme of *Bacillus* sp. No. 202-1 is not inhibited by EDTA or PCMB treatment.[465]

B. ISOPULLULANASE

Isopullulanase (pullulan 4-glucanohydrolase EC 3.2.1.57) splits α-1,4-glucosidic linkages in pullulan (see Figure 2), but has hardly any activity on starch. It frees isopanose as the main product.[463] Isopullulanase has been found only from *A. niger* ATCC 9642, where it is produced at 30°C as an intracellular enzyme.[463] Its temperature optimum is at 30 to 40°C, and the pH optimum is between 3.5 and 4.0.[463] The enzyme is stable from pH 4.0 to 7.0.[463]

C. AMYLOLYTIC ENZYMES OF *BACILLUS STEAROTHERMOPHILUS* KP 1064, TRS40, AND *THERMOACTINOMYCES VULGARIS* STRAINS 42 AND R-47

Amylase produced by *T. vulgaris* 42[279] and R-47,[281] and *B. stearothermophilus* KP 1064[492] is able to hydrolyze several substrates and also pullulan. *B. stearothermophilus* enzyme can hydrolyze pullulan, amylopectin, soluble starch, amylose, α- and β-limit dextrins, α- and β-cyclodextrins, phenyl-α-D-maltoside, maltotriose, and maltopentaose.[239] These enzymes liberate panose from pullulan by hydrolyzing 1,4-linkages. *T. vulgaris* R-47 amylase is able to attack some 1,6-glucosidic bonds in partial hydrolysates of pullulan as well.[281] Both these activities are related to one catalytic site,[281] which consists of six subsites.[493] *B. stearothermophilus* KP 1064 enzyme is composed of dimers.[239] These enzymes have been classified as α-amylases, though they are difficult to categorize. Their properties are presented in Table 3.

The *B. stearothermophilus* TRS40 enzyme produces mainly panose from pullulan, and small amounts of glucose and maltose are simultaneously produced.[464] The enzyme has a molecular weight of 62,000 and exhibits optimum activity at pH 6.0 and at 60 to 65°C.[464] The enzyme designated as neopullulanase retains about 90% of its activity after incubation at 60°C for 1 h.[464] This enzyme is presumably a new class of pullulan-degrading enzymes. The other panose-liberating enzymes have high activities on starch, whereas neopullulanase has only limited activity.

IX. GLUCOAMYLASE

Glucoamylase (1,4-α-D-glucan glucanohydrolase EC 3.2.1.3) is an exoacting carbohydrase which splits 1,6-, 1,4-, and also some 1,3-glucosidic linkages of α-glucans.[490] It yields β-D-glucose from the nonreducing end of the substrate. Many glucoamylases hydrolyze pullulan. Glucoamylase is produced by many fungi, but only by a few bacteria, including *B. stearothermophilus,*[228] *C. thermohydrosulfuricum,*[466] *Flavobacterium* sp.,[382] and *Halobacterium sodomense.*[491] Glucoamylases are commercially important in the degradation of starch, production of glucose and fructose syrups, and ethanol. Many of the fungal enzymes mentioned in Table 9 are used in industrial production.

A. MULTIPLE FORMS OF GLUCOAMYLASE

The number of glucoamylases per strain ranges between one and five (see Table 9). Several mechanisms have been presented to describe their formation. Pazur et al.[492] state that forms of *A. niger* enzymes are "isoglycoenzymes", which have different amounts of carbohydrates. Almost all the fungal glucoamylases are glycoproteins, and varying amounts of carbohydrate moieties could give rise to some of the observed multiple forms. The pH of the culture medium determines the pH stability of the *Aspergillus awamori* var. *fumeus*-1-B42 glucoamylase.[493] Three forms of *A. awamori* var. *kawachi* enzyme were produced selectively in different media.[494] Hayashida[494] reports that in the presence of zinc glucoamylase may be degraded to smaller forms by proteases. The molecular weights of these enzymes, designated GAI, -I′, and -II, are 90,000, 83,000, and 57,000, respectively.[495] Glucoamylase I was converted to smaller molecular weight forms resembling GAII by stepwise degradation *in vitro* with fungal acid protease and glycosidases.[495] An enzyme similar to GAI′ was obtained by degradation with acid protease and α-mannosidase.[496] Though properties of the degraded forms were not exactly similar to the actual forms, these results show that in some cases multiple glucoamylases can be formed by limited proteolysis. This conclusion is supported by the properties of the protease negative *A. awamori* var. *kawachi* mutant, which produces glucoamylase as a protein having a molecular weight of 250,000,[497] from which GAI-like protein was derived *in vitro* by proteolytic degradation.[459] Glucoamylase activities in *A. niger* mutants are directly proportional to their proteolytic activity.[498] An enzyme resembling the smaller molecular weight glucoamylase of *A. niger* NRRL 3122 is produced by the action of proteinase VIII of *B. subtilis* on GAI.[499] Different forms of *A. niger* glucoamylase result from different mRNA splicing.[500] It is not clear if multiple forms in other *A. niger* strains are due to proteolysis, which can produce *in vitro* quite similar forms, or if the mechanisms are similar to those in industrial strain.

Amino terminal amino acids of *Rhizopus* sp. glucoamylases are different.[501] Proteolytic degradation of high molecular weight $Gluc_1$ with papain and chymotrypsin caused formation of enzymes resembling smaller glucoamylases, $Gluc_2$ and $Gluc_3$, of this strain.[502] *Saccharomyces diastaticus* 5106-9A enzyme is composed of two nonidentical subunits,[503] whereas enzyme from strain YIY2-12D is monomeric.[504] *S. diastaticus* has three loci for glucoamylase genes,[505] but *A. niger, A. awamori,* and *Rhizopus oryzae* have only one.[506-508] Based on the results summarized previously, it is clear that various forms of glucoamylase are apparently results of several mechanisms: mRNA modifications, limited proteolysis, variation in carbohydrate contents, and several structural genes.

B. CLONING GLUCOAMYLASE GENES

The number of genes coding for glucoamylase varies among strains. *A. awamori* and *A. niger,* which each produce two forms of glucoamylase, have only one structural gene.[504,511] *S. diastaticus* has three polymorphic genes which are located in different linkage groups.[509] Hybridization of a probe oligonucleotide, synthesized based on knowledge of a partial amino

acid sequence, with only one fragment in *R. oryzae* chromosomal DNA, indicated the presence of only one gene.[513]

The gene coding for *A. awamori* glucoamylase has been cloned and sequenced.[504,514] It has four intervening sequences, and these had to be removed before it could be expressed in yeast.[515,516] Production in *S. cerevisiae* also requires yeast promoter and termination signals.[515] Under ideal conditions with the constructed vector, 25 to 30% of the total supernatant protein produced in *S. cerevisiae* was glucoamylase.[515] The deduced amino acid sequence contains a putative signal peptide of 24 amino acids.[515] Distiller's yeast used for commercial ethanol production cannot ferment starch. Introduction of starch-degrading activities in yeast is advantageous because costly starch hydrolysis prior to fermentation could be avoided. The engineered yeast strains described above are not suited for commercial production because of poor ethanol tolerance and enzyme production.[516]

Glucoamylases G1 and G2 of *A. niger* are coded by the same structural gene.[504] The gene contains four introns, and one more intron occurs when the messenger for glucoamylase G2 is formed by splitting 169-bp fragment from mRNA.[504] The C-terminus of the protein has a new coding frame from this cleavage site on.[504] These glucoamylases have a signal peptide of 18 amino acids and 6 additional amino acids which are removed proteolytically.[514]

S. cerevisiae and *S. diastaticus* are closely related yeasts. The greatest difference between them is the ability of *S. diastaticus* to carry out fermentation on starch. *S. cerevisiae* produces sporulation-specific glucoamylase from the *SGA* gene.[517] *S. diastaticus* has three unlinked genes for glucoamylases.[509,518] There are two ways to designate genes involved in *S. diastaticus: STA* and *DEX* systems. *DEX1* is allelic to *STA2*, and *DEX2* is allelic to *STA1*.[519] In *S. cerevisiae*, three fragments hybridize with *STA1* gene.[520] These authors suggest that three separate regions in the *S. cerevisiae* chromosome have possibly recombined and formed a functional gene.[520] The above results suggest that a number of genes could have arisen from translocation of *STA* genes in the chromosome.[520]

The *STA1* gene of *S. diastaticus* has also been cloned[521] and sequenced.[522] The gene encodes a large propeptide of 778 amino acids, from which 2 subunits of the mature protein are cleaved proteolytically.[522] There are still 21 amino acids to be removed from the carboxy terminus of the shorter fragment. The gene has been cloned in *Schizosaccharomyces pombe*, where glucoamylase is produced with the same properties as in *S. cerevisiae* and in the donor strain.[523,524] Production of glucoamylase in *S. diastaticus* is controlled by *MAT* function.[525]

Cloned *STA3* gene has a similar restriction map as *STA1* and is highly homologous, as indicated by hybridization studies.[522] The restriction map of cloned *STA2*[526-528] is similar to maps for other *STA* genes. Some *S. cerevisiae* strains carry genes *STA10*[529] and *INH1*,[530] which inhibit glucoamylase expression. The sporulation-specific *SGA* gene of *S. cerevisiae*, which has been cloned,[524] is repressed by the *STA10* gene.[524] Control of glucoamylase production in *S. cerevisiae* is primarily at the level of mRNA accumulation.[527]

The *R. oryzae* SAM0034 glucoamylase gene has been cloned in *E. coli* and *S. cerevisiae*.[509] The nucleotide sequence shows that this gene contains 4 introns and a signal sequence for 25 amino acids.[509] To express this gene in yeast, intervening sequences had to be removed and 5′- and 3′-noncoding sequences were added.[509] *S. fibuligera* HUT7212 glucoamylase gene has been cloned in *S. cerevisiae*[528] and sequenced.[529] The yeast cells secrete glucoamylase and can ferment starch.[528] The enzyme has a signal sequence of 28 residues.[529] Comparison of the known glucoamylase sequences showed five highly homologous segments, one of which seems not to be essential for the amylolytic activity since *Saccharomyces* glucoamylases are lacking it.[529] No apparent homology was found with α-amylases.[489] *Rhizopus* and *Aspergillus* sequences seem to be the most related.[489]

C. PROPERTIES OF GLUCOAMYLASES

Fungal glucoamylases are glycoproteins which contain carbohydrates up to 28% by

weight (see Table 9). Carbohydrates may be necessary for maintenance of the active three-dimensional conformation of some enzymes.[598] They are linked O-glycosidically to serine and threonine residues. Structures of carbohydrate moiety in some glucoamylase have recently been reviewed.[9] The enzyme of *S. castellii* CBS 2863 does not contain a carbohydrate moiety.[324] *R. niveus* enzyme called D[591] is probably the same as fraction C in the same strain.[599] Glucoamylase is an extracellular enzyme in fungi, in *Flavobacterium* sp. tightly cell-bound,[408] and in *C. thermohydrosulfuricum* both intracellular and membrane-bound.[466]

Glucoamylases have optimal pH in the acidic range and are rather stable for prolonged periods at acidic pH values (see Table 9). Though many glucoamylases have temperature optimum at or near 60°C, only the enzymes produced by *C. thermohydrosulfuricum*,[466] *Humicola lanuginosa*,[576] and *Thermomyces lanuginosa*[597] can be considered as thermostable. Glucoamylase from *H. sodomense* requires over 1 *M* salt concentration to be maximally active,[491] and its temperature optimum is a function of salt concentration.[491] K_m values differ for different forms of glucoamylases. Raw starch adsorptivity and digestibility varies also, so that usually the highest molecular weight forms hydrolyze raw starch effectively, and the others not at all or with lower K_m values. The N-terminal portion of *R. oryzae* glucoamylase is proposed to be responsible for adsorbtion to starch and the C-terminal portion to be involved catalytically in starch degradation.[530] The C-terminal part seems to be responsible for introducing raw starch-degrading activity of *A. niger* glucoamylase.[546] The C-terminal region present in *A. niger*, *S. fibuligera*, and *R. oryzae* sequences is lacking from *S. cerevisiae* and *S. diastaticus* glucoamylases[529] and implies that different parts of the sequence can be involved in different glucoamylases to govern raw starch-binding and hydrolysis.

Molecular weights of glucoamylases vary greatly from 20,000 to 306,000 (see Table 9), the largest of which is stated to be formed of two protomers.[594] The enzyme of *S. diastaticus* is composed of two nonidentical subunits.[503,518] SDS-PAGE, which is mostly used for molecular weight determination, gives only estimates for glycoproteins.[600,601] Heavy metal ions inhibit glucoamylases. Role of the Ca^{2+} varies. For example, the enzyme from *Rhizopus delemar* is inhibited by high concentrations of Ca^{2+},[588] whereas glucoamylase from *Schizophyllum commune* is stimulated by it.[311] Calcium decreases stability of enzymes from *A. oryzae*[556] and *Endomycopsis fibuligera* IFO 0111.[572] Starch stabilizes enzymes produced by *A. niger*,[551] *C. thermohydrosulfuricum*,[466] *H. lanuginosa*,[576] and glycerol enzyme from *Candida tsukubaensis*.[562] Based on chemical modification studies of *A. niger* glucoamylase, tryptophan residues have been proposed to be essential for enzymatic activity.[601,602] One residue was thought to be involved in the binding of substrate and another in catalytic activity.[601] Trp-120 is responsible for binding of substrate and might maintain structural integrity necessary for catalysis.[602] It has been speculated that the Trp-120 could have analogous function as Trp-83 in Taka-amylase A.[602]

X. α-GLUCOSIDASE

α-Glucosidases or maltases (α-D-glucoside glucohydrolase EC 3.2.1.20) are distributed widely among microorganisms. They hydrolyze 1,4- and/or 1,6-glucosidic linkages of short saccharides, which are formed by action of other amylolytic enzymes, and liberate α-D-glucose units from the nonreducing end. α-Glucosidases are usually present with other amylolytic enzymes. Enzymes of *Penicillium oxalicum*,[608] *P. purpurogenum*,[609] and *Tetrahymena pyriformis*[610] hydrolyze amylose or starch as well. Many of these enzymes also display transglycosylation activity; examples are the enzymes produced by *Bacillus* sp.,[611] *B. amyloliquefaciens*,[612] *B. cereus*,[613] *B.subtilis*,[614] *Lentinus edodes*,[581] *S. cerevisiae*,[615] and *S. pombe*.[616]

Oligo-1,6-glucosidase or isomaltase (dextrin 6-α-D-glucanohydrolase EC 3.2.1.10) is an enzyme similar to α-glucosidase. It degrades only α-1,6-linkage at the nonreducing end

TABLE 9
Properties of Glucoamylases

Strain	T_{growth} (°C)	Number of enzymes	pH optimum	Temp. optimum	pH stability[a]	Thermostability	K_m (mg/ml)[b]	Mol wt[c]	Isolectric point	Carbohydrate content (%)	Inhibitors	Miscellaneous
olomyces rouxii RLL 5866	28	1	4.5	60			15.8 16.8 (2) 27.6 (3)	55,600 (1)				
ergillus sp.		2	4.6			50% 68°C, 43 min		70,000 (1) 81,000 (3)				Trademark Agidex 3000®; producer Glaxo Laboratories Inc., both forms have similar properties
ergillus sp. N-2	35	4	3.5—4.5		5.5—6.5 30°C, 40 h							
ergillus awamori O 4033	30	1	4.5	60	5.0—9.0	Below 50°C		88,000 (1) 83,700 (3)	3.7		Al^{3+}, Hg^{2+}, Pb^{2+}	
awamori X-100								102,000 (3)		17		
awamori var. *wachi*		3	3.8		2—9 30°C, 20 h	95% 65°C, 30 min 20% 70°C, 30 min		90,000 (1)	3.55	7.6	Cu^{2+}, Hg^{2+}, KCN	EDTA has no effect
			3.8		2.5—8.5 30°C, 20 h			83,000 (1)	3.45	4.6		
			4.0		4—7 30°C, 20 h			57,000 (1)	3.28	1.0		
awamori var. *kachi* HF-15	32				2—8 30°C, 20 h	100% 60°, 15 min 35% 70°, 15 min		250,000 (1)		24.3		
ergillus batatae 61				4—5			0.12 0.49 (5)					
ergillus candidus	28	2	4—5	60				75,000 (1)				
ik var. *aureus* 19b			4—5	60				60,000 (1) 72,000 (1,4)				
ergillus clavatus VU-GD1	30		5.2	40								
ergillus hennebergi chwitz	35	2	4.0—5.0	70		100% 60°C, 15 min 40% 70°C, 15 min		60,000 (4)	3.9			Only the GAII is glycoprotein
			4.0—5.0	70		100% 60°C, 15 min 40% 70°C, 15 min		72,000 (4)	3.9			

llus niger 152	30		4.0	52								
r		2	4—5	60	4—11 RT, 1 h	100% 60°C, 30 min 20% 70°C, 30 min		78,000 (1) 70,000 (4)	3.2	13	Gluconolactone	Trademark Diazyme®, producer Miles Laboratory, Elkhardt, U.S.
			4—5	60		100% 60°C, 30 min 20% 70°C, 30 min		60,000 (1) 59,000 (4)	4.0	18		
r		2	4—5	60				79,000 (1)				Producer Anis Starch Product, Ahmedabad, India
			4—5	60				72,000 (1)				
r		2	4—5	60				79,000 (1)				Producer Sarabhai Chemicals, Baroda, India
			4—5	60				60,000 (1)				
r		1	4—5	60				60,000 (1)				Producer Maize Products, Ahmedabad, India
r		2						85,000 (1) 82,000 (2)	3.6;4.0	17—19		Trademark AMG 200 L®, producer Novo Industries, Bagsvaerd, Denmark; gene has been cloned; the two forms arise from different mRNA splicing
								61,000 (1,2) 46,000 (3)	4.2	22		
r 16/132			4.5	60				43,150 (4)				
r ATCC 13496	34	2	5	50	4—6 30°C, 24 h	Below 60°C						
r B77			4.5	50—55	4.0—7.0 4°C, 24 h	100% 45°C, 60 min 25% 55°C, 60 min					Cu^{2+}	Ca^{2+}, Fe^{2+}, Mn^{2+} stabilize
r CISIR-N_4	28		4.0—4.3	70								
r IMDCC No.			4.5	70	2.0—9.0 40°C, 30 min	100% 50°C, 30 min 10% 70°C, 30 min	0.023% 1.42 m*M* (5)	63,000 (4)	4.0		Ag^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, p-hydroxymercuribenzoate	Starch and glycerol stabilize; polyvalent anions and metal chelators stimulate
r NRRL 330		2						99,000 (3) 112,000 (3)				Different forms are isoglycoenzymes
r NRRL 3122	30	2	4.5	60		100% 60°C, 30 min		85,300 (4)				
			4.5	60		80% 60°C, 30 min		77,600 (4)				

TABLE 9 (continued)
Properties of Glucoamylases

Strain	T_{growth} (°C)	Number of enzymes	pH optimum	Temp. optimum	pH stability[a]	Thermostability	K_m (mg/ml)[b]	Mol wt[c]	Isolectric point	Carbohydrate content (%)	Inhibitors	Miscellaneous
er van Tieghem RI 1105	35	2	4.7	65	3—6 4°C, 24 h	100% 60°C, 10 min 15% 70°C, 10 min	72.5 m*M*	69810 (1)		17	Ag^+, Hg^{2+}	
			3.5	60	1—2 4°C, 24 h	100% 40°C, 10 min 87% 60°C, 10 min	24.8 m*M*	89130 (1)		25	Ag^+, Hg^{2+}	
gillus oryzae 245	21		3.6—6.5	50—60			12.82					ATCC No. 9376
zae	30	3	4.5	60	4.0—7.0 40°C, 30 min	100% 40°C, 30 min 25% 60°C, 30 min	13.3	76,000 (4)	5.6			
			4.5	50	4.0—7.0 40°C, 30 min	100% 40°C, 30 min 15% 60°C, 30 min	6.25	38,000 (4)	5.6			
			4.5	40	4.0—7.0 40°, 30 min	100% 40°C, 30 min 10% 60°C, 30 min	1.11	38,000 (4)	5.6			
zae	32	4	5.5—6		3—5 4°C, 30 min	100% 40°C, 20 min 10% 60°C, 20 min						
			5—7		3—5 4°C, 30 min	100% 40°C, 20 min 15% 60°C, 20 min						
			4.5—6.5		3—5.5 4°C, 30 min	100% 40°C, 20 min 15% 60°C, 20 min		69,000 (3)	3.3			
			5		4—5.5 4°C, 30 min	100% 40°C, 20 min 0% 60°C, 20 min						
zae RIB-40	30	3					2.50		4.0			
							2.70		3.9			
							2.0		3.6			

hoenicis Staley 5		4.6	60		85% 25°C, 72 h 55% 25°C, 192 h		63,600 (3) 62,000 (4)		17		Producer A.E. Staley Manufacturing Co, Illinois, U.S.
aitoi	2	4.5	50	2.5—7.5 37°C, 60 min	Below 50°C	0.42% 0.077% (1) 0.19% (2) 0.37% (3) 0.33% (4) 2.7 m*M* (5) 1.2 m*M* (6) 0.17 m*M* (7) 0.16 m*M* (8) 0.14 m*M* (9)	90,000 (1,5)	3.85	18.8	Hg^{2+},Mn^{2+}, Pb^{2+}	Trademark Molsin,● producer Seishin Pharm. Co., Japan
		4.5	50	2.5—7.5 37°C, 60 min		0.92% 0.146% (1) 1.65% (2) 1.25% (3) 0.73% (4) 3.0 m*M* (5) 1.4 m*M* (6)	70,000 (1,5)	3.86	12.6		
eobasidium pullu-ns A-124	25	5.75	50		100% 45°C, 10 min 0% 60°C, 10 min						
ndida tsukubaensis BS 6389	29	2.4—4.8	55	2—7 20°C, 24 h	100% 30°C, 30 min 15% 50°C, 30 min		56,000 (1)				Glycerol stabilizes
phalosporium char-ola Lindau	28	5.4	60				69,000 (4)		6.6		
phalosporium ei-horniae 152	46	4.2	45—62		100% 62°C, 15 min 50% 70°C, 15 min		26,800 (1)	3.25	28		Phosphate anion activates

TABLE 9 (continued)
Properties of Glucoamylases

Strain	T_{growth} (°C)	Number of enzymes	pH optimum	Temp. optimum	pH stability[a]	Thermostability	K_m (mg/ml)[b]	Mol wt[c]	Isolectric point	Carbohydrate content (%)	Inhibitors	Miscellaneous
sporium resinae	30	2	3.5—4.0	60	4.0—7.0 37°C, 20 h	Below 55°C	0.007 0.007 (2) 0.003 (3) 0.8 (5) 0.32 (6,7) 0.30 (8) 0.08 (9) 0.58 (13) 5.4 (14) 6.3 (15)	70,000 (1)	4.6	4.6	Fe^{3+}, Hg^{2+}, Mn^{2+}	
			3.5	60	4.0—6.0 37°C, 20 h	Below 48°C	0.007 0.007 (2) 0.003 (3) 0.6 (5) 0.3 (6) 0.2 (7,8,9) 150 (13) 9.9 (14) 11.0(15)	82,000 (1)	4.5	8.2		
ridium thermohy-ulfuricum 39E			4—6	75	5—6 60°C, 60 min	100% 65°C, 60 min 15% 75°C, 60 min	0.41 0.32 (1)					DSM 2229; ATCC 33743; substrate stabilizes. Tolerates ethanol 10% (v/v)
ophora cerebella L 11E			4.0—4.5			0% 65°C, 10 min	0.42% (1) 0.03% (2) 0.63% (5)	48,000 (4)			Hg^{2+}	
cium rolfsii AHU 7	27		4.0	65	3.0—7.0 40°C, 60 min	90% 55°C, 10 min 7% 70°C, 10 min						
lfsii IFO 4878			4.5	40—60	2.0—9.0 2°C, 72 h						Fe^{3+}, Sn^{2+}	ATCC No. 24964
mycopsis sp. 20-9								53,000 (1)	3.8	8.5		

ycopsis bispora		2	5.5	65	6—7 45°C, 30 min	95% 50°, 10 min 30% 60°, 10 min	0.5	47,800 (4)				
			5.5	60	5—6 45°C, 30 min	100% 50°, 10 min 35% 60°, 10 min	2.5	50,100 (4)				
ycopsis capsu- 311/1	30		4.5	40—50	4.2—8.0 29°, 36 h							
ycopsis fibuli- 241	21		5.2—6.5	50—60			7.05					
uligera IFO 0111	30		4.8—5.0		4—7.5 30°C, 24 h	100% 50°C, 10 min 65% 70°C, 10 min	20	55,000 (3)	4.8—5.5	9.5	Hg^{2+}	Ca^{2+} enhances thermal inactivation
uligera R1	32		5—6	50—60				57,000 (1)				
uligera Y1	32	1	5.5	60	4.0—9.0 40°C, 30 min	100% 40°C, 30 min 30% 60°C, 30 min		58,000 (4)				IFO 1665
hia parasitica						18% 50°C, 10 min						Trademark Sure Curd®, producer Pfizer Inc., Milwaukee, Wisconsin, U.S.
asidium uligenum	30	2	5.0—5.6	55	5.0—5.6 35°C, 24 h	100% 40°C, 30 min 25% 55°C, 15 min		60,000 (1)				
			4.8—5.3	50	4.8—5.3 35°C, 24 h	75% 40°C, 30 min 18% 50°C, 15 min		60,000 (4)				
bacterium sp.	28		5.5—6.5		5.8—7.2 20°C, 48 h	below 55°C, 10 min	0.142 m*M* (10)				EDTA	Cell-bound; cyclodextrinase; calcium stabilizes
bacterium *mense* 6	35		7.5	65 (1,4 *M* NaCl) 75 (3,9 *M* NaCl)								ATCC No. 33755
cola lanuginosa /1	37	2	4.9 6.6	65—70	5—10 RT 24 h	100% 50°C, 30 min 52% 70°C, 30 min						Starch stabilizes
nus edodes (Berk) .			4.5	50	3.5—5.5 30°C, 20 h	Below 40°C, 15 min		55,000 (1)	4.1		Al^{3+}, Hg^{2+}, Pb^{2+}	
nyces *onenkoae* S 5608	25		4.5	50	4—6.5 30°C, 1 h	Below 50°C, 4 h	16.2 0.36 (5) 0.35 (17)	81,500 (1)	6.1		Glucose	

TABLE 9 (continued)
Properties of Glucoamylases

Strain	T_{growth} (°C)	Number of enzymes	pH optimum	Temp. optimum	pH stability[a]	Thermostability	K_m (mg/ml)[b]	Mol wt[c]	Isolectric point	Carbohydrate content (%)	Inhibitors	Miscellaneous
ascus kaoliang . sp. F-1	30	2	4.5	50	4.0—8.5 30°C, 24 h	100% 50°C, 10 min 30% 55°C, 10 min	0.41% 0.36% (1) 0.61% (2) 1.6% (3) 0.1% (5)	48,000 (1)			Hg^{2+}, Th^{4+}, $KMnO_4$	
			4.7	60	4.0—7.5 30°C, 24 h	100% 50°C, 10 min 85% 55°C, 10 min	0.008% 0.02% (1) 0.003% (2) 0.009% (3) 0.1% (5)	68,000 (1)			Fe^{3+}, Th^{4+}, $KMnO_4$	
r rouxianus IFO 3	30	2	4.6	55	4.0—8.0 30°C, 24 h	100% 50°C, 15 min 15% 55°C, 15 min	0.46 (1) 0.74 (2) 1.11 (3)	59,000 (1)	8.4		Hg^{2+}, Pb^{2+}, Tris	
			5.0	55	4.0—7.5 30°C, 24 h	100% 50°C, 15 min 15% 60°C, 15 min	0.29 (1) 0.67 (2) 1.54 (3)	49,000 (1)	8.4	2.74		
ospora crassa			5.2			50% 55°C, 12 min	4 m*M* (5)	82,000 (1)				
ilomyces varioti U 9471	28		4.5		4.5—10 4°C, 24 h	100% 50°C, 15 min 5% 65°C, 15 min	0.001 m*M* 0.04 m*M* (1) 1.2 m*M* (5) 0.26 m*M* (6) 0.14 m*M* (7) 0.15 m*M* (8) 0.12 m*M* (9)	69,000 (1,4)				

							59 m*M* (14) 12 m*M* (15) 22 m*M* (16)					
illium oxalicum 7000	30	2	5.0	55—60	3.0—6.5 30°C, 20 h	100% 55°C, 15 min 40% 60°C, 15 min		84,000 (3)	7.00	3.93	Hg^{2+}, Tris	ATCC No. 20592
			4.5	60	3.0—6.5 30°C, 20 h	100% 55°C, 15 min 55% 60°C, 15 min		86,000 (3)	7.45	3.22		
a polymorpha ker 106	28		4								Ba^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, potassium hexacyanoferrate, EDTA	
ularia oryzae ura 6908	28	1	6.5	50—55	5.5—7.0 30°C, 24 h	100% 40°C, 15 min 25% 50°C, 15 min		94,000 (1)				
pus sp.		3	4.5—5.0		4.0—8.0 37°C, 60 min	100% 50°C, 5 min	0.007% 0.003% (2) 0.006% (3) 3.17 m*M* (5) 1.31 m*M* (6)	73,600 (1) 74,000 (3)	8.7	11.0	Hg^{2+}, Mn^{2+}, Pb^{2+}	Trademark Gluczyme,® producer Amano Pharmaceutical Co., Nagoya, Japan
			4.5—5.0		4.0—8.0 37°C, 60 min	100% 50°C, 5 min		59,000 (1) 58,600 (3)	8.7	9.3		
								66,000 (1)	8.8	13.5		
			4.5—5.0		4.0—8.0 37°C, 60 min	100% 50°C, 5 min		61,400 (3)				
opus delemar			4.5				0.062% 6.6 m*M* (5)	100,000 (3)		13	Ca^{2+} at high concentrations	Producer Miles Chemical Co., Elkhardt, Indiana, U.S.
delemar		3			4.0—8.5 40°C, 30 min	Below 45°C, 30 min	6.1 μ*M* (2) 240 μ*M* (3) 1.9 m*M* (5) 0.59 m*M* (6) 0.23 m*M*	61,000 (1)				Producer Shin-Nihon Kagaku Kogyo Co., Japan

TABLE 9 (continued)
Properties of Glucoamylases

Strain	T_{growth} (°C)	Number of enzymes	pH optimum	Temp. optimum	pH stability[a]	Thermostability	K_m (mg/ml)[b]	Mol wt[c]	Isoelectric point	Carbohydrate content (%)	Inhibitors	Miscellaneous	Ref.
							(7) 0.16 m*M* (8) 0.12 m*M* (9) 17 m*M* (14) 14 m*M* (15) 7.4 μ*M* (2) 300 μ*M* (3) 1.9 m*M* (5) 0.57 m*M* (6) 0.21 m*M* (7) 0.14 m*M* (8) 0.11 m*M* (9) 47 m*M* (14) 13 m*M* (15)						
					4.0—8.5 40°C, 30 min	Below 45°C, 30 min		70,000 (1)					
					4.0—8.5 40°C, 30 min	Below 45°C, 30 min	0.3 μ*M* (2) 0.8 μ*M* (3) 2.0 m*M* (5) 0.64 m*M* (6) 0.26 m*M* (7) 0.17 m*M* (8) 0.16 m*M* (9)	78,000 (1)					

								18 m*M* (13) 40 m*M* (14) 14 m*M* (15)					
eus		5	5.5		3—8.5 25°C, 24 h		1.2 m*M* (5) 0.4 m*M* (6) 0.27 m*M* (7) 0.20 m*M* (8) 0.10 m*M* (9)	60,000 (1)	8.45	14.9			Producer Seikagaku Kogyo Co., Ltd., Japan; glucose, lactose, and gluconolactone stabilize
			5.0						9.1	12.7			
dosus UB 378	28	1	4.8	60	5.0—8.0 15°C, 24 h	100% 45°C, 15 min 50% 60°C, 15 min		71,000 (1)			Hg^{2+}		EDTA has no effect
aromyces visiae 536	25					94% 45°C, 60 min		150,000 (4)					Gene has been cloned
aromyces taticus 5-9A	28					50% 50°C, 195 min		41,000 (2) 3,400 (2)					Gene has been cloned; composed of two nonidentical subunits
staticus J132b		2	5.4	50			10 m*M* (5)						
staticus J1741c		3	5.4	50			10 m*M* (5)						
staticus SPX-1C			5.5	60		52% 50°C, 60 min 10% 60°C, 60 min	18 m*M* (5)	306,000 (4) 186,000 (1)	4.1				Gene has been cloned
ophyllum mune	26		4.8	45				20,000 (4)			Hg^{2+}, EDTA		Ca^{2+} stimulates.
mmune IFO 4928	30	1	5.0			100% 50°C, 10 min 45% 60°C, 10 min		66,000 (1)					
anniomyces vius	30		4.5	50	4—6 30°, 1 h		22.22	117,000 (4)					
uvius UCD 54-83	30		5.0	50	4.0—8.0 30°, 24 h	88% 50°C, 30 min	12.67 0.72 m*M* (5)	155,000					
anniomyces cas- 1402	21		4.2—5.5	40—50		100% 50°C, 30 min 1% 60°C, 15 min		10.31		0			ATCC No. 26077; is not glycoprotein

TABLE 9 (continued)
Properties of Glucoamylases

Strain	T_{growth} (°C)	Number of enzymes	pH optimum	Temp. optimum	pH stability[a]	Thermostability	K_m (mg/ml)[b]	Mol wt[c]	Isoelectric point	Carbohydrate content (%)	Inhibitors	Miscellaneous
castellii Capprioti 3S 2863	28	2	6.0	60		100% 60°C, 30 min 70% 75°C, 30 min		90,000			Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, EDTA, PCMB, α-gluconolactone	
			6.0	60		100% 60°C, 30 min 70% 75°C, 30 min		45,000				
ermomyces lanugi-osa ML-M	50	1		70		100% 50°C, 6 h 60% 60°C, 6 h	0.046 0.022 (3) 0.114 (4) 0.833 (5) 0.556 (6)	55,000 (1) 57,000 (4)		10—12		
ichoderma viride 3S 354.44	29		5.0—5.5		4—7 2°C	Below 60°C, 10 min						

RT means room temperature.
Substrate is indicated by number in parentheses; starch is without number; substrates are (1) amylose; (2) amylopectin; (3) glycogen; (4) dextrin; (5) maltose; (6) maltotriose; (7) maltotetraose; maltopentaose; (9) maltohexaose; (10) α-cyclodextrin; (11) β-cyclodextrin; (12) γ-cyclodextrin; (13) pullulan; (14) isomaltose; (15) panose; (16) nigerose.
Method used to measure mol wt is given in parentheses; (1) SDS PAGE; (2) amino acid or DNA sequence; (3) sedimentation analysis; (4) gel filtration; (5) amino acid analysis.

of short chain substrates. It has been found from *Bacillus thermoglucosidius* KP1006[612] and *B. cereus* ATCC 7064,[619] and probably from *B. amyloliquefaciens* ATCC 23844,[614] which has two glucosidases.

Saccharomyces species require the presence of at least one of the five closely related unlinked polymeric *MAL* loci (*MAL1-MAL4, MAL6*) to ferment maltose.[620] These enzymes are subject to both maltose induction and glucose repression. The *MAL1* and *MAL6* locus of *Saccharomyces* species have been cloned[621,622] and shown to consist of three genes.[622,623] The other *MAL* regions also have this kind of gene cluster.[623] *MAL2* or *MALS* is the structural gene for α-glucosidase, *MAL1* or *MALT* codes for maltose permease, and *MAL3* or *MALR* is the positively acting regulatory element.[624] *MAL64*, which maps near *MAL63*, is another *trans*-acting gene which controls maltase and maltose permease production.[625] The *MAL1* locus is highly homologous, and the genes are organized similarly to the *MAL6* locus.[622] The cloned *MAL* genes indicated that *MALR* also requires maltose for induction on a multicopy plasmid, and maltose transport can be a rate-limiting factor in expression of *MALS* and *MALT*, at least in the early stages of induction.[626] A strain having the *MAL12* gene of the *MAL1* locus produces enzyme which has a thermostability different from that produced from *MAL62*.[624] The *MAL6S*, *MAL6R*, and part of the *MAL6T* genes have been sequenced.[627,628] The *MAL6S* gene confers production of enzyme having molecular weight of 68,100[627] and *MAL6R)* protein with calculated molecular weight of 55,000.[628] The regulatory protein has in the N-terminus sequence isologous to a consensus sequence for cystein-zinz-associated DNA-binding fingers of other fungal transcriptional regulatory proteins.[628] The regulatory region is between 320 and 380 bp upstream from the transcription initiation codon of *MAL6S*.[629]

A. PROPERTIES OF α-GLUCOSIDASES

α-Glucosidases appear often in several forms as glucoamylases and often in the same microbes. Extra- and intracellular forms from *A. niger* are probably due to different genes,[630] but enzymes from *Thermoascus aurantiacus* seem to be "isoglycoenzymes" because they have very similar properties, except for molecular weights and carbohydrate contents.[658] Anti-α-glucosidase against extracellular *A. niger* enzyme did not recognize the intracellular counterpart.[630] Extra- and intracellular enzymes of *B. licheniformis* NCIB 8549 are similar.[638] The exact nature of different forms of α-glucosidase has not been elucidated in any strain.

Most α-glucosidases are intracellular enzymes (see Table 10), though some strains have both extra- and intracellular forms. They all have pH optima near pH 7 or acidic region (see Table 10). This is the case even for the enzyme from the alkalophilic *Bacillus alcalophilus*.[611] Some of the enzymes are stable also in alkalic region, e.g., *B. alcalophilus*[611] and *B. licheniformis*[637] enzymes. None of the known enzymes have a very high temperature optimum, although enzymes of *Bacillus* sp. KP1035[633] and *T. aurantiacus*[658] are rather thermostable.

Molecular weights of α-glucosidases vary between 12,000 and 160,000 and carbohydrate contents between 0 and about 50% (see Table 10). The lowest molecular weight is exceptionally small since the cloned and sequenced *S. carlsbergensis* α-glucosidase has a molecular weight of 68,100.[627] α-Glucosidases are inhibited by certain carbohydrates, e.g., turanose, glucose, and heavy metal ions. EDTA does not usually have any effect on activity. Ca^{2+} stimulates the enzyme of *B. brevis*[635] and inositol, and albumin stabilizes the *Pseudomonas fluorescens* enzyme.[648] Substrate specifities of α-glucosidases, which have been extensively studied, have not been discussed here because a thorough review covering that subject has appeared only recently.[7]

XI. SUMMARY

Amylolytic enzymes are produced by an extremely wide variety of microorganisms.

TABLE 10
Properties of α-Glucosidases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp. optimum	pH stability	Thermostability	K_m (mg/ml)[b]	Mol wt[c]	Isoelectric point (d)	Carbohydrate content (%)	Inhibitors	Miscellaneous
rgillus awamori	30	I	5.0	55	4.5—6.0	below 45°C		125,000 (1)	5.60		Cu^{2+}, Hg^{2+}, Pb^{2+}	
4033		I	5.0	55		below 45°C		140,000 (1)	5.60			
		I	5.0	55		below 45°C		130,000 (1)	5.60			
iger	30	I	5.0			12%, 65°C, 20 min	1.04 (5) 1.49 (17) 1.85 (5)	95,000 (4) 63,560 (3)				630, 631
		E	5.0			50%, 65°C, 20 min	2.22 (17)			14		
ryzae			4.2—4.6	55							Cu^{2+}, Hg^{2+}	Trademark Takamaltase®
llus sp. KP 1035	55	E			5.6—9.0 60°C, 24 h	95%, 70°C, 20 min 17%, 75°C, 20 min						
lcalophilus sp. *odurans* ATCC 91	37	E	7.0	40	7.4—8.6 40°C, 1 h							Alkalophilic strain; previously *Bacillus* sp.
nyloliquefaciens CC 23350	37	I	6.8	39	6.2—7.0 11°C, 24 h			27,000				Membrane-associated enzyme
nyloliquefaciens CC 23844	37	I	5.3	40				27,000 (4)	4.6			
revis RL B-4389	30	E	6.5	48—50	5.0—7.0 40°C, 3 h	100%, 50°C, 3 h 15%, 60°C, 30 min	5.8 (5)	52,000 (4)	5.2		Al^{3+}, Cd^{2+}, Cu^{2+}, Co^{2+}, Fe^{3+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, glucose, p-nitrophenyl-α-D-glucoside, Tris	Ca^{2+} stimulates
ereus	30	I	6.8—7.3	40	6.8—7.3 30°C, 20 h	100%, 30°C, 15 min 30%, 45°C, 15 min	5.55 (5)	12,000 (1)	4.5		Cu^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Tris, turanose, DHT, PCMB, rose bengal	
rculans CC 9995	30		7.0	40								Mostly intracellular enzyme; previously *Bacillus amylolyticus.*
cheniformis B 6346	37		6.0	50	5.5—10.0	100%, 37°C, 60 min 30%, 55°C, 60 min		63,000 (1)			Glucose, glucono-δ-lactone, Tris	Intracellular in logarithmic growth phase and extracellular in stationary phase; Identical forms

heniformis B 8549	30	E	6.0	40	5.5—8.0 RT, 20 min	100%, 30°C, 60 min 0%, 50°C,		160,000 (4) 66,000 (4)			Glucose, glucose-δ-lactone	
			6.0		5.5—6.5 RT, 20 min	60 min 100%, 30°C, 60 min		63,000 (1)			Glucose, glucose-δ-lactone, Tris	
	30	E		40		15%, 50°C, 60 min						
otilis P-11	37	E	6.0	45	5.5—6.5, 45°C, 90 min	80%, 45°C, 2 h 50%, 50°C, 2 h	5 (5) 1 (6) 10 (14)	33,000 (4)	6.0		Glucose, rose bengal, Tris	
da tropicalis *japonica*			4.0		3—7 0°C, 8d	80°, 60°C, 10 min 5%, 65°C, 10 min						
us edodes (*k.) Sing.*		I	5.0	50	4.0—6.0, 30°C, 20 h	Below 40°C, 15 min		51,000 (1)	4.6		Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}	
r javanicus IFO	30	I	4.6	55	4.0—7.0 30°, 20 h	100%, 45°C, 15 min 40%, 55°C, 15 min	0.675 (5)		8.6		Cu^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+}, turanose, Tris	
cemosus IFO	23	I	4.0—6.0	50	4.0—7.0, 30°C, 20 h	100%, 45°C, 15 min 5%, 60°C, 15 min	0.916 (5) 0.991 (6) 9.253 (14) 2.813 (20)	114,000 (3)		4.1	Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, turanose, Tris, DHIT	
uxii			3.5	50—60			0.37 (5)			40		Mostly cell wall-bound enzyme
illium oxalicum 7000	30	I	3.5	50	3.5—7.0, 30°C, 20 h	100%, 37°C, 15 min 45%, 55°C, 15 min	1.23 (5) 0.50 (6) 2.32 (14)				Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, turanose	
rpurogenum 6022	30	I	3.0—5.0	50	5.5—7.0, 30°C, 20 h	100%, 37°C, 15 min 5%, 50°C, 15 min	0.69 (5)	120,000 (1)	3.20		Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, turanose, Tris	
omonas oderamosa 5		I	5.0	50	4—8, 40°C, 30 min	70%, 55°C, 20 min 10%, 60°C, 20 min	0.77% (1) 0.61 (5) 0.74 (6) 0.60 (17)				Ag^{+}, Cu^{2+}, Hg^{2+}, glucose, turanose	ATCC number 21262
uorescens W	30	I	7.0			10%, 50°C, 2 min	0.035 (17)				Ag^{+}, Cu^{2+}, Hg^{2+}, Tris, PCMB, turanose, NEM	Inositol and albumin stabilize
haromyces sbergensis 11	30	I	6.7—6.8				16.6 (5) 0.31 (17)	63,000 (4)				

TABLE 10 (continued)
Properties of α-Glucosidases

Strain	T_{growth} (°C)	Location[a]	pH optimum	Temp. optimum	pH stability	Thermostability	K_m (mg/ml)[b]	Mol wt[c]	Isoelectric point (d)	Carbohydrate content (%)	Inhibitors	Miscellaneous
romyces isiae		I	6.3	42			28.6 (14) 2.9 (18) 50.0 (19)	52,000 (4)				
		I	6.3—6.8	42			14.3 (5) 6.2 (6) 20.0 (16) 0.8 (18) 2.8 (20) 11.7 (21) 16.6 (22)	52,000 (4)				
		I	6.8—7.1	36			71.4 (5,22) 14.3 (6) 100.0 (14) 3.6 (18) 14.3 (20) 30.3 (21)	52,000 (4)				
evisiae 448-	30	I	7.0				110 (5) 0.21 (17)	66,000 (1) 67,000 (3)	5.65			
		I	7.0				105 (5) 0.18 (17)	66,000 (1) 67,000 (3)	5.92			
evisiae 510-	30	I	7.0				99(5) 0.22 (17)	66,000 (1) 67,000 (3)	5.64			
evisiae 727-	30	I	7.0				93 (5) 0.20 (17)	66,000 (1) 67,000 (3)	5.63			
evisiae 1412-	30	I	7.0—7.5			8%, 51°C, 7 min		68,500 (4)				Two forms
		I	7.0—7.5			8%, 51°C, 2 min		64,700 (4)				
romyces us 5		I	6.8		6—7.8		0.28 (17)	85,000 (3)			Ag^+, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, PCMB, iodoacetate, Tris, histidine, quinine, benzylamine	
os 4471	27	I	4.6—5.0	40	3.6—6.6 27°C, 20 h	100%, 37°C, 15 min 0%, 50°C, 15 min	7.7 (5) 8.2 (6) 6.0 (7,8) 16.5 (14) 11.1 (15) 11.5 (16) 8.7 (18) 3.6 (20)	27,000 (4)		50		

osaccharo-es pombe	25	I	4.0—4.4	45	3.2—6.8 30°C, 20 h	100%, 42°C, 15 min 40%, 50°C, 15 min						3 fractions on chromatography	
tococcus genes		I	6.6—6.8	37		0%, 50°C, 6 min					Ag^+, Cu^{2+}, Fe^{2+}, Hg^{2+}, Zn^{2+}, Tris, PCMB, iodoacetate, erythritol		
hymena pyri-is W		E	4.0				18.5 mg/ml (3) 2.5 (5) 33.0 (14) 1.1 (17)	110,000 (4)		2.8	Turanose		
nascus antiacus	45	I					0.245 (17)	140,000 (4)	4.2	16.2		Four forms	
he		I				95%, 70°C, 40 min	0.236 (17)	140,000 (4)	4.2	14.1			
		I					0.250 (17)	145,000 (4)	4.2	28.7			
		I					0.230 (17)	145,000 (4)	4.2	31.5			

(E) extracellular; and (I) intracellular enzymes.

Substrate is indicated by number in parentheses. Starch is without number. Substrates are (1) amylose; (2) amylopectin; (3) glycogen; (4) dextrin; (5) maltose; (6) maltotriose; (7) maltotetraose; (8) malt (9) maltohexaose; (10) α-cyclodextrin; (11) β-cyclodextrin; (12) γ-cyclodextrin; (13) pullulan; (14) isomaltose; (15) panose; (16) nigerose; (17) p-nitrophenyl-α-D-glucoside; (18) phenyl-α-glucoside; (19) glucoside; (20) phenyl-α-maltoside; (21) turanose; and (22) sucrose.

Method used to measure mol wt is given in parentheses; (1) SDS PAGE; (2) amino acid or DNA sequence; (3) sedimentation analysis; (4) gel filtration; (5) amino acid analysis.

Properties of these enzymes vary and are somewhat linked to the natural environment occupied by the producing organisms. Physicochemcical properties show that starch can be degraded under very variable conditions. Degradation of starch usually requires cooperation of several enzymes because only a few enzymatic activities can degrade it wholly alone, thus, starch-degrading microbes usually have several amylolytic activities.

Among all the amylolytic systems, the genetics of α-amylase in *B. subtilis* is best known. α-Amylase production is regulated by several genetic elements, many of which have synergistic effects. Genes for α-amylase have been cloned from several strains. Sequences show some highly conserved regions. Genes have been cloned and sequenced also for the other amylolytic enzymes. Multiple forms of fungal glucoamylases seem to be the result of a variety of mechanisms, including heterogeneous glycosylation, limited proteolysis, multiple modes of mRNA splicing, and the presence of several structural genes.

Molecular biological studies have greatly extended our knowledge of amylolytic enzymes and their production. However, production levels have usually not been as high as has been assumed possible, and the properties of the enzymes are less than ideal. Some of this can be overcome by optimum use of good host organisms, expression/secretion vectors, and site-directed mutagenesis. The molecular biology of amylolytic enzymes has been and continues to be a valuable system for studying problems such as the mechanisms of gene regulation, secretion, and structure-function relationships in proteins.

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