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REVIEW

Characteristics and Production of Thermostable α-Amylase

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Abstract

Thermostable α-amylases have application in a variety of industrial processes and enzymes from a substantial number of thermophilic bacteria and fungi have been screened and characterized to varying degrees. The characteristics of these enzymes are summarized in this review. The genetics of α-amylase production in *Bacillus subtilis* is reviewed and classical and recombinant DNA approaches to increasing α-amylase production are discussed.

Index Entries: Thermostable α-amylases; α-amylases, characteristics and production of; genetics, of α-amylase production in *Bacillus subtilis; Bacillus subtilis*, genetics of α-amylase production in; recombinant DNA, of α-amylase; DNA, of α-amylase, classical and recombinant techniques in.

I. Introduction

Alpha-amylase or 1,4-α-D-glucan glucanohydrolase (E.C.3.2.1.1), is an enzyme that degrades starch by cleaving internal α-1,4-glucosidic bonds, resulting in a rapid loss of viscosity. The enzyme has a number of applications in commercial processes, including thinning and liquification of starch in the alcohol, brewing, and sugar industries. It is also used for desizing of fabrics before dyeing in the textile industry. The primary products of α-amylase digestion of starch are malto-

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TABLE 1
Amylases

α-Amylase (dextrinogenic, liquifying)	β-Amylase (saccharogenic)	Glucoamylase (amyloglucosidase)
1. Hydrolyze α-1→4 glucosidic bonds	———	———
2. Produce products with α configuration at C_1 of the reducing glucose unit	Product products with β configuration at C_1 of the reducing glucose unit	———
3. Possess an endo-attack mechanism[a]	Possess an exo-attack mechanism	———
4. Rapidly decrease ability of amylase to stain blue with iodine[b]	Slowly decrease ability of amylase to stain blue with iodine[b]	———
5. Rapidly decrease the viscosity of starch solution	Slowly decrease the viscosity of starch solution	———
6. Can bypass α-1→ 6 branch points	Cannot bypass α-1→6 branch points.	Probably cannot bypass α-1→6 branch points
7. Produces α-maltooligosugars	Produces β-maltose	Produces β-glucose

[a]An exo-α-amylase from *Pseudomonas stutzeri* has been reported (*2, 3*).
[b]Relative to number of bonds split, as measured by increased reducing power (*1, 4*).

oligosugars of varying lengths; thus the enzyme is different from β-amylase, an exohydrolase producing maltose, and glucoamylase, an exohydrolase producing glucose. The distinguishing features of these enzymes are presented in Table 1. The substrate specificity and intermediate and limit digest products of α-amylase vary somewhat according to its source (*1*).

α-Amylase has been isolated from a wide variety of bacterial, fungal, plant, and animal sources. Since enzymes with thermostable properties are particularly useful in the industrial applications listed above, this report will deal with those enzymes with thermostable properties, isolated from thermophilic fungi, and particularly thermophilic bacteria. For reviews on microbial amylases in general, the reader is referred to several sources (*5–7*). In addition, topics relevant to genetic engineering for enhanced production of α-amylase will be discussed.

II. Thermophilic Microorganisms

There are a surprising number of microorganisms that survive, and are in fact adapted to, life at temperatures from 60 to 100°C (*8*). These creatures elaborate enzymes that are, in general, more active at high temperature and more thermostable than those produced by their mesophilic counterparts (*9*). In addition, there is a direct, though not absolute, correlation between the temperature optimum for growth and the thermostability of the enzyme in question. Therefore, organisms with elevated temperature ranges for growth are good candidates for production of thermostable α-amylase.

In general, eukaryotic microorganisms do not live above 60–65°C, while prokaryotes exist even in boiling or superheated water (*5*). Not surprisingly, of the α-amylases that have been characterized, those from thermophilic bacteria are generally more stable than those from thermophilic fungi that have lower maximum temperatures for growth.

III. Bacterial α-Amylases

By far the most work that has been done with thermophilic α-amylases has been with bacteria of the genus *Bacillus*, in which nearly every representative produces α-amylase. *Bacillus thermocatenalatus*, which grows at 78°C, is reported not to hydrolyze starch, though it ferments dextrin (*10*). The presence of α-amylase in another thermophilic genus, *Thermomicrobium* (*11*), has not been reported. The genus *Thermus* is widespread (*12–16*), and one group reports small amounts of amylase in seven strains tested (*17*), whereas another failed to detect the enzyme (*18*), possibly because the plates on which the bacteria were grown and assayed contained a high concentration of starch (1%) that the low level of α-amylase might not have been able to degrade detectably. In the characterization of new *Thermus* species, *Thermus flavus*, Saiki et al. (*15*) reported that neither *Thermus flavus* nor *Thermus aquaticus* YT-1 (*12*) hydrolyze starch to produce acid, though degradation of starch was not monitored directly.

A summary of properties from thermophilic *Bacillus* species is presented in Table 2. The α-amylase with the highest temperature for optimal activity comes from *Bacillus licheniformis*, reported to be 76–92°C, depending on the laboratory, the conditions of the assay, and perhaps the individual strain (see Table 2 for references). Interestingly, this species is only a moderate thermophile, having a temperature optimum for growth of 45°C, and a maximum temperature for growth of only 55°C. This enzyme, which is marketed commercially to the starch industry, has a broad pH range for activity, is highly thermostable in the presence of starch, and has a lower requirement for Ca^{2+} than many bacterial α-amylases (*32, 33*).

Tamuri et al. (*31*) screened several hundred thermophilic fungi and over a thousand thermophilic bacteria for thermostable α-amylase. They compared the properties of α-amylase from *Bacillus licheniformis* with those of several newly isolated strains of *Bacillus stearothermophilus*. Although the optimal temperature for activity is somewhat lower for the *B. stearothermophilus* enzyme, the pH range extends more to the acidic side and the enzyme shows superior thermostability properties at low pH and low $CaCl_2$ concentrations in comparison to *B. licheniformis*. This enzyme may be superior in the production of high fructose corn syrup if the initial liquification of starch can be carried out at pH 4.5 so that pH adjustment is not necessary prior to glucoamylase digestion.

Other *Bacillus* α-amylases show marked thermostability as well (e.g., *B. caldolyticus*). The thermostability data for *B. coagulans* and *B. stearothermophilus* reported by Campbell (*24, 25*) are impressive, but are included here only for the sake of completeness since other workers have been unable to repeat this work (*28–30*).

Of interest because of the extreme conditions under which they function are two acidophilic α-amylases from *B. acidocaldarius* (*19*) and *B. sp.* 11–15 (*20*) with pH

TABLE 2
Thermostable Bacterial α-Amylases

Bacillus species	T_{growth}, C°	Enzyme activity		Enzyme stability	
		T, °C	pH	*T* % remaining activity	pH % remaining activity
B. acidocaldarius	60	75 (sharp drop > 80)	3.5	75°C, $t_{1/2}$ = 2 h 60°C, $t_{1/2}$ = 5 d	Stable below pH 4.5
B. sp. 11–15	65 pH 3–4	70	2.0	pH 2 70°C, 10′, 60% 30′, 6%	pH 1–2 15′, 65°C no loss of activity
B. coagulans	55 35	60–70 45–55		60′, 90°C 94% 8%	
B. stearothermophilus	55 35	60–70 45–55	6.5–8.0	90% 10%	
B. stearothermophilus		60	5.5–7.5	3 h, 90°C 50%	
B. stearothermophilus		55–70	4.6–5.1	24 h 70°C 100% 20 h 85°C 71%	
B. stearothermophilus		65–73	5–6 (65°C)	90°C, $t_{1/2}$ = 20′ 75°C, stable	7–11 (26°C)
B. stearothermophilus	I 98% 55 II 2% 43 I 100%	70 (I,II) 70 (I)	4–6 (I,II) 4–6 (I)	I 72% 80°C, 10′,II 48% I 68% 90°C, 30′ All activity lost	I 47% II 25% I 43% 5–12 (all forms)
B. stearothermophilus				Stable at 70°C w/Ca^{2+}, BSA; unstable at 60°C wo/CA, BSA	6.5–9.0 (25°C)
B. stearothermophilus	60	75–80	4.0–5.2	$t_{1/2}$ 90°C, pH 6.0 0 m*M* $CaCl_2$ > 60′ 1 m*M* $CaCl_2$ > 60′ 85°C, pH 4.55 22.5% starch 1 m*M* $CaCl_2$, 30′	
B. licheniformis *B. subtilis*		92 70	5–7 in presence of 0.5% starch 4 m*M* $CaCl_2$	$t_{1/2}$ 93°C, 25 h 98°C, 400′ 103°C, 100′	pH 6.5 2 m*M* $CaCl_2$ 30% starch

TABLE 2 (*continued*)

MW	K_m	V_{max}	Comments	Ref.
68,000 SDS PAGE Sedimentation	0.8–0.9 mg/mL	35 μmole maltose/ min/mg protein	80–90% extracellular; requires Ca^{2+}, Mg^{2+} for optimal activity; stability of enzyme at high T function of protein conc.; requires (maltose)$_{1-4}$, or starch to induce; produced late in culture.	*19*
54,000 SDS-PAGE	1.64 mg/mL		Ca^{2+}, EDTA do not affect thermostability	*20*
				21, 22
				23
15,500			Ca^{2+} enhances activity	*24, 25*
48,000 sedimentation			Stability enhanced by Ca^{2+}, reduced by EDTA	*26, 27*
46,000 44,000 43,000 41,000	0.77 mg/mL (all forms)			*28*
44,000 41,000 Sedimentation gel filtration				
			*Were unable to reproduce Cambell's work (*21, 22*). See also (*29*). *T* stability function of pH, [starch], [protein], [Ca^{2+}] enzyme cold sensitive.	*30*
90,000 SDS-PAGE			Low requirement for Ca^{2+}, thermostable at low pH; enzyme not pure	*31*
			Enzyme has low requirement for Ca^{2+}	*32, 33* technical info. supplied with Termamyl

(*continued*)

TABLE 2 (*continued*)
Thermostable Bacterial α-Amylases

Bacillus species	T_{growth}, C°	Enzyme activity		Enzyme stability	
		T, °C	pH	*T* % remaining activity	pH
B. licheniformis	50	76 (pH 8)	5–8	5′, 60°, 100% 70°, 100% 80°, 45%	6–11 (24 h 25°C)
B. licheniformus CUMC305		91 (pH 9.0)	9.5	60′, 70°, 100%	4–10
B. coagulans CUMC512		85 (pH 8.5)		40′, 75°, 100% 60′, 60°	Unstable at higher *T* and when held for longer time
YT-P/*B. caldolyticus*	72	70 50% activity at 90	5.4	60′, 80°, 0% 35′, 90°, 0%	Protein not pure; may be proteolytic contaminant
V-2 (probably not *Bacillus*)	65	70	6–7	60′, 90°, 50%	
Bacillus NoA-40-2	40	55 inactive at 70°C	10–10.5	30′, 50°, 100% (5 m*M* $CaCl_2$) 30′, 60°, 0%	
Bacillus No. 38-2			4.5, 9.0	15′, 60° 85% 10 m*M* Ca^{2+} 35%, Ca^{2+}	5–10.5
Acinetobacter		I, 50	$+Ca^{2+}$ 60°C; 7.0		7–8 (9 w/Ca^{2+})
		II 50	60°C; 7.0		7–8 (9 w/Ca^{2+})
B. licheniformis		90	5–9	$t_{1/2}$ 95° 30′	pH 7.0 5 m*M* $CaCl_2$;
N.Z.	70	75	5–8	95° 20′	no starch

optima of 3.5 and 2.0, respectively, and alkalophilic α-amylases from *B. sp.* NoA 40-2 (*41*) and 38-2 (*42*) with pH optima over 10.

Clearly α-amylases have evolved in species of widely differing habitats, and as extracellular enzymes they must have very distinctive properties. In fact, the α-amylases show such a wide variety of properties that this family would make an interesting case for the study of structural determinants that affect the optimal conditions for enzyme activity.

Thermophile V-2, isolated from Japanese hot springs, appears to be a non-*Bacillus* α-amylase producer, since it is gram-negative and does not produce spores (*39, 40*). The amylase from this organism is extremely thermostable, although its temperature optimum for activity is not as high as that from *B.*

TABLE 2 (*continued*)

MW	K_m	V_{max}	Comments	Ref.
22,500 gel filtration			4 bands on electrophoresis that reran in the same position	*34*
			Thermal stability is substrate-dependent	*35*
	Ultrafiltration experiments suggest that Ca^{2+} enhances stability of a high molecular weight form of α-amylase, which upon extensive washing or depletion of Ca^{2+} becomes a lower molecular weight form that is less active			*36–38*
50,000 SDS-PAGE	At 50–70°C 1.4×10^{-2}–2.2×10^{-2} mol glycosidic bonds/L		pI = 9.2 10 m*M* Ca^{2+}, 50 m*M* Na^{+} enhance activity; bound Ca^{2+} present, resistant to denaturing agents	*39, 40*
			Extremely alkalophilic enzyme; does not require starch for induction	41
			Enzyme not purified stabilized by Ca^{2+}	*42*
55,000 SDS-PAGE 65,000 SDS-PAGE	Required 0.2–0.6*M* NaCl or KCl for full activity, inactivated by H_2O			*43*
	Enzyme has a low requirement for Ca^{2+} for activity and thermostability			Mainzer et al. (in preparation)

licheniformis. A gram-negative thermophile from New Zealand hot springs, characterized in this lab (Mainzer et al., in preparation) also produces a thermostable, thermoactive α-amylase.

IV. α-Amylases from Thermophilic Actinomycetes and Fungi

The enzymes of the filamentous bacteria, the *Actinomycetes*, have been less completely characterized than the *Bacillus* α-amylases; however, the enzyme from *Thermoactinomyces vulgaris* has a temperature optimum comparable to many of the *Bacilli*. Table 3 presents the scanty data available.

Although thermophilic fungi are the source for glucoamylases used commercially, extensive characterization of the α-amylases from these organisms has not

TABLE 3
α-Amylases from Actinomycetes and Thermophilic Fungi

Organism	Enzyme activity: *T*(opt.), °C	Enzyme activity: pH opt.	Enzyme stability	K_m	Comments	Ref.
Actinomycetes						
Thermoactinomyces vulgaris	70	5	60°C, pH 5 stable (10 m*M* $CaCl_2$)		Hydrolyzes pullulan	*44, 45*
Thermoactinomyces vulgaris		120′ 70°C pHs 50%	65–70°C maximum; most stable at pH 5.9			*46*
Thermonospora viridis		5.9–7.0				*47*
Micromonospora vulgaris					Distinct *T* optimum 45–50°C for maximal production	*48*
Thermonospora curvata	65	5.5–6.0	5′, 90°, 60% +BSA, 5% 60′, 90°, 0%; substrate has little effect	0.39 mg/mL	Starch, amylose, maltose, maltotriose are good inducers	*49, 50*
Thermonospora vulgaris	60	6	pH: 3.8, 5′, 0% activity 7.0, 60′, 100% 9.0, 30′, 75% *T*: 60′, 60°C, 50% 5′, 80°C, 0% $CaCl_2$, starch, and β-ME did not enhance *T* stability		Induced by 2% starch or solid $CaCO_3$; *T* (opt.) for production of α-amylase is 55°C	*51, 52*

Fungi					
Aspergillus niger	60	5.5–6.0	30′, 60°C, 82% 60′, 60°C, 36% 30′, 60°C, 20%	Ca^{2+} required for enzyme stability at high pH, substrate for stability at high temp.	*53*
Aspergillus niger (II)			15′, 40° 100% (pH 5.0) 50°, 95% 60°, 88% 70°, 27% 80°, 0%	Optimal stability between pH 3.4–6.4 Two α-amylases produced	*54–56*
Humicola insolens, H. languinosa, Mucor pusillus, Chaetomium thermophile, Thermoascus aurantiacus, Malbranchea pulchella, T. crustaceus	45–50	6–7			*57*
Chaetominum thermophile, Humicola grisea, H. insolens, H. lanuginosa, H. stellata, Malbranchea pulchella, Mucor miehei, M. pusillus, Talaromyces thermophiluts, Torula thermophila					*58*
Humicola lanuginosa, H. grisea, H. brevis, H. grisea var *thermoidea*				*Humicola lanuginosa* produced by far the most enzyme	*59*
Absidia corymbifera, A. ramosa, Aspergillus fumigatus, Mucor pusillus, Renizopus microsporius, Thermoascus crustaceous, Thermomyces lanuginosus					*60*
Mucor miehei, M. pusillus				*M. pusillus* prolific producer	*61*
Papulaspora thermophila					*62*

been reported. Table 3 includes a list of actinomycetes and fungi that have been shown to produce α-amylases. The temperature optimum for activity and temperature stability of most of these enzymes has not been reported. That of *Aspergillus niger* has a temperature optimum of 60°C, about 10°C lower than most of the thermophilic *Bacilli*, and the enzyme is only somewhat stable at temperatures higher than 60°C.

V. General Properties of α-Amylases

In amylolytic micoorganisms most of the α-amylase activity is found in culture media and a relatively small amount is intracellular or membrane bound (*63*). This is consistent with a function in degrading a high molecular weight extracellular polymer into units that are small enough for subsequent enzyme activity or transport into the cell. Intracellular forms of the enzyme have been found (*64*), but both genetic (*65*) and pulse-chase experiments employing several inhibitors of protein synthesis during the chase (*66*) suggest that the intracellular α-amylase forms are precursors to mature secreted forms of the enzyme.

In general, α-amylase is produced relatively late in culture, after the culture has achieved stationary phase. Production of α-amylase occurs when cells are grown in the presence of starch, maltose, or maltose oligomers, and it is likely that this represents induction of the enzyme. In *Bacillus stearothermophilus*, the level of α-amylase production is inversely proportional to growth rate, using several sugars, and inducible by starch, maltose, and maltodextrins (*67, 68*). Little α-amylase is present when cells are grown in glucose, and there is evidence that in *Bacillus licheniformis* and *Bacillus subtilis* α-amylase production is subject to catabolite repression (*69, 70*).

For the most part, thermophilic bacterial α-amylases do not differ greatly from their mesophilic counterparts in molecular weight (most are 45,000–60,000 daltons) and general amino acid composition. The molecular weights of some have been compared using SDS polyacrylamide gel electrophoresis and sedimentation or gel filtration (*19, 28*) suggesting that these enzymes are monomeric. However, Robyt and Ackerman (*71*) have studied multiple forms of α-amylase from *Bacillus subtilis* and have shown that there is a 96,000 dalton, zinc-containing tetramer, and also trimer, dimer, annd monomeric forms of the enzyme. The specific activity of trimer, tetramer, and aggregated forms is higher than the smaller dimers (48,000 dalton) and monomers. All of these multimeric forms run at their native molecular weights on SDS gels. These data, along with the lower molecular weight forms reported for *B. licheniformis* (*34*) and *B. caldolyticus* (*37, 38*) suggest that some bacterial α-amylases may be composed of subunits. Of interest is the observation that mouse α-amylases expressed in pancreas, salivary gland, and liver are specified by mRNAs 1577, 1663, and 1774 nucleotides in length (72); each one is long enough to specify a ~50,000 dalton polypeptide.

Most α-amylases require Ca^{2+} for stability at high temperature (see Table 2, also *73*) and there is evidence that the enzyme contains a bound Ca^{2+} (*39, 40*),

which is responsible for maintaining the integrity of the enzyme (*37, 38, 74*). The *B. licheniformis* enzyme is notable in having a relatively low requirement for Ca^{2+} in the medium at high temperature (*32, 33*), and similarly *B. stearothermophilus* is thermostable even in the absence of Ca^{2+} (*31*). Some of the bacterial α-amylases have been reported to be stabilized by the substrate at high temperature. These are listed in Table 2.

Walker and Hope (*75*) compared the action of several α-amylases on fine starch granules in suspension with their activity on soluble starch. Human salivary and pig pancreatic α-amylases were relatively effective at degrading maize starch graunles; *B. subtilis* α-amylase degraded this insoluble starch slowly, and *Aspergillus oryzae* α-amylase was completely ineffective in digesting maize starch granules.

Of interest is the fact that although there are marked similarities in bacterial α-amylases, the antibody against *B. subtilis* α-amylase does not cross-react with antibody against *B. amyloliquefaciens* α-amylase (*76*). The *N*-terminal amino acid sequence of *B. subtilis var. amylosacchariticus* has been published (*77*). Matsuzaki et al. (*78*) reported that *Bacillus subtilis* Marburg α-amylase has 9% carbohydrate, whereas *B. subtilis* Natto and *B. subtilis var. amylosacchariticus* have no carbohydrate.

Urabe et al. (*79*) have reported that acetylation of amino groups in *Bacillus subtilis* α-amylase results in reduced stability at low temperature, but enhanced stability at high temperature. Enzyme activity is reduced somewhat with increasing alkylation.

VI. Assays

There are three basic kinds of assays that are used for detecting α-amylase activity. In the first case, the release of reducing sugars from a starch substrate is measured either with 3,5-dinitrosalicylate or by a colorimetric copper reaction (described in Ref. *1*). In the second case, the disappearance of the specific reaction between iodine and starch after amylase digestion is measured (*1, 80*). This reaction can also be utilized for detecting α-amylase activity in agar plates (*81*) or after gel electrophoresis (*82–84*). In the third type of assay, a chromogenic group is attached to the substrate and the release of this substrate into the soluble fraction is monitored by a change in optical density (*85, 86*).

VII. Purification Schemes

In addition to conventional protein purification protocols (ammonium sulfate fractionation, ion exchange, and gel filtration chromatography), advantage is often taken of specific interaction between α-amylases and substrates for affinity-type purification. Cornstarch is used for adsorbing the enzyme, (see for example, refs. *30, 34,* and *39*), though some enzymes, for example, the one from *Bacillus acidocaldarius*, do not adsorb to solid starch (*19*). Loyter and Schramm (*87*), de-

scribe formation of a glycogen α-amylase complex for purification. Here one does not have to worry about differences in grain size affecting the binding capacity as with insoluble starch.

A number of affinity columns have been prepared using α-amylase inhibitors. Burrill et al. (*88*) purified rat pancreatic and salivary α-amylases using Bay g5421, a compound containing an unsaturated cyclitol unit that may be a transition state analog and is an inhibitor of α-amylase. Silvanovich and Hill (*89, 90*) coupled a Schardinger β-dextrin (cycloheptaamylase) to Sepharose 6B to isolate cereal α-amylase. Takeuchi (*86*) used concanavalin A to separate human amylase isozymes and Buonocore et al. (*91*) employed a wheat germ albumin that inhibits α-amylase from various sources.

VIII. Genetics that Govern α-Amylase Production in Bacillus

Most of the work concerning the genetics of α-amylase production has been done in *Bacillus subtilis*. This work may be instructive in terms of suggesting strategies for optimizing production of α-amylase in other thermophilic *Bacillus* species using similar mutation and selection and DNA transformation methodology. In addition, *Bacillus subtilis* α-amylase overproducers may be excellent hosts for the synthesis and excretion of α-amylase encoded by genes of thermophilic microorganisms that have been introduced using recombinant DNA technology.

Green and Colarusso (*92*) first demonstrated that the α-amylase gene could be transferred between closely related strains of *Bacillus* using whole cell DNA to transform recipients. Interestingly, the α-amylase gene of the donor repressed synthesis of the recipient α-amylase; when the transformant was mutated so that the donor gene no longer functioned, the recipient allele could once again function. Therefore, the transformation did not result in replacement of the recipient allele, but did silence it. Isono (*93*) reported the transformation of *Bacillus stearothermophilus* amylase mutants with *B. stearothermophilus* DNA.

B. subtilis Marburg produces about five times more α-amylase than *B. natto*. Yamaguchi et al. (*94*) showed, using reciprocal transformation experiments, that this is because the promoter of *B. natto* α-amylase, *amyR2* is a stronger one than that in *B. subtilis* Marburg, *amyR1*. The controlling regions, *amyR2* and *amyR1*, are closely linked to the α-amylase structural gene, *amyE*. Matsuzaki et al. (*95*) demonstrated recombination within the structural gene resulting in hybrid proteins. A third control region, *amyR3*, from *B. subtilis var. amylosaccharitícus* is phenotypically similar to *amyR2* (*96, 97*).

Sasaki et al. (*98*) showed that certain tunicamycin resistant strains of *B. subtilis* produced five times more α-amylase than the parent. When DNA from these strains was used to transfrom sensitive *B. subtilis* to tunicamycin resistance, there was an absolute correlation between tunicamycin resistance and α-amylase overproduction. This tunicamycin resistant locus, *tmr7* maps near *amyR* (*98*).

In addition, there are unlinked genes that regulate the production of α-amylase, and in some cases, levels of other extracellular enzymes. Mutations, described alternatively as *papM* (100), *amyB* (101), and *sacU* (102), which appear to be iden-

tical (103), increase production of several extracellular enzymes, including α-amylase, proteases, and levansucrase, as well as altering transformability and flagellar synthesis. There is an analogous allele described in *B. subtilis var. amylosaccharitcus* (104), *papS1*, as well as another regulator of α-amylase levels, *amyS1*, from the same organism.

Hitotsuyanagi et al. (*105*) isolated two other types of unlinked α-amylase overproduction mutants, one from a nitrosoguanidine mutagenesis in which cycloserine resistance was selected (A2). In these instances, the drug resistance could be separated from the regulatory mutants, indicating that drug resistance and amylase overproduction were mutations at different sites.

Hitotsuyanagi et al. (*105*) and Yoneda (*104*) showed that by combining these unlinked mutant alleles for α-amylase overproduction in the same strain by successive transformations, their effect is synergistic and hyperproducers of α-amylase result. Direct mutagenesis of these strains leads to still higher producers of α-amylase. Where tested, the structure of the enzyme appears to be unaffected. Although genetic analysis has not been done to confirm the presence of all alleles, the resulting strain produces 1600 times more α-amylase than the original parent. This is an impressive example of how significantly α-amylase production can be enhanced in the absence of recombinant DNA techniques that increase gene dosage. Strain T2N26, the result of this program, produces the equivalent of 2.5 mg/mL pure protein in its culture medium. Amino acid labeling indicates that this is 7–10% of protein produced in a 20-min labeling period (*105*). If the enzyme is highly stable, α-amylase may represent an even higher percentage of total protein at steady state. Not surprisingly, these hyperproducers of α-amylase appear to have higher levels of specific α-amylase mRNA as judged from in vitro translation experiments (*106, 107*).

In *Bacillus licheniformis* relatively little has been published concerning α-amylase overproduction, but Saito and Yamamoto (*69*) have isolated mutants that seem immune to catabolite repression and produce significantly more α-amylase without the lag that is seen in wild-type cells.

IX. Transformation and Cloning of α-Amylase

Alpha-amylase genes have been cloned by a number of groups. Young et al (*72*) described the cloning of mouse α-amylase genes of pancreatic, salivary gland, and liver origins. The results are interesting from a molecular biology standpoint since a single structural gene is transcribed into two different mRNAs with different leaders in salivary gland and liver.

Nomura et al. (*108*) have cloned the structural gene of *Bacillus subtilis* (*amyE*) in *B. subtilis* using the temperate phage ρ11. *AmyE* is closely linked to *aroI*, an auxotrophic marker, so that using $aroI^{-}$ $amyE^{-}$ recipients, aro^{+} transformants were selected and scored for α-amylase activity. ρ11-AA4 and ρ11-AA15 both contained an α-amylase gene that was cotransformed or cotransduced with the *aro* marker at a high frequency.

Yoneda et al. (*109*) cloned the *B. amyloliquefaciens* α-amylase gene in ϕ3T of *Bacillus subtilis*. Since *Bacillus* genes from species other than *Bacillus subtilis* often do not transform *B. subtilis* efficiently, this interspecies transformation was done in the following way. *B. amyloliquefaciens* DNA (*amy*$^+$) was ligated into *B. subtilis* phage ϕ3T and used to transform a *B. subtilis thr*$^-$ *amy*$^-$ ϕ3T lysogen. *B. subtilis* uncloned DNA (*thr*$^+$, *amy*$^-$) was mixed with the ϕ3T/*B. amyloliquefaciens* DNA and *thr*$^+$ cells, which may also have been transformed by the ϕ3T/*B. amyloliquefaciens* DNA, were screened for α-amylase activity. In this case, the homology between the lysogenic ϕ3T and the recombinant ϕ3T DNA was to allow recombination to carry the foreign gene into the *B. subtilis* genome. In fact, $7/10^5$ transformed cells synthesized α-amylase and when the ϕ3T was induced to undergo a lytic cycle, five of the seven bacteriophage strains carried the *B. amyloliquefaciens* gene for α-amylase.

Palva et al. (*110*) cloned the *Bacillus amyloliquefaciens* α-amylase gene into B. subtilis using the *Bacillus* plasmid cloning vector pUB110. Kanamycin resistant transformants of an α-amylase mutant recipient were screened for α-amylase activity. Nucleic acid sequence work has completely characterized the leader sequence. The authors claim that 50% of the protein synthesized in the *Bacillus subtilis* transformant is α-amylase, which is 500× more than their wild-type strain. They speculate that this is caused by the efficient α-amylase promoter and the high copy number of the plasmid.

CPC has cloned α-amylase genes from *Bacillus megaterium*, and *Bacillus coagulans* into pBR322 and λ vectors in *E. coli* (*111*). Production is enhanced as much as 2× and 100× over parent strains of *B. megaterium* and *B. coagulans*, respectively, when pBR322 is the vector. Cornelis et al. (*112*) cloned the *Bacillus coagulans* α-amylase gene in λ and pBR322 vectors showed that in *E. coli* the enzyme accumulates in the periplasmic space. Shinomiya et al. (*113*) transformed a *Bacillus subtilis* restriction and modification mutant lacking α-amylase with uncloned DNA from thermophile V2. They screened for and found α-amylase production among colonies that had been transformed for an auxotrophic marker. Shinomiya et al. (*114*) have characterized the α-amylase produced by these transformants. It has immunologic, electrophoretic mobility, and temperature optimum properties like the DNA donor, thermophile V2. The transformed α-amylase is not inactivated by EDTA. The *amyV2* structural gene has been localized in transformants between *pyrA* and *metC*. When *amyV2* and *amyE*, the *Bacillus subtilis* structural gene, are both present, both function and show properties of thermophile V2 and *Bacillus subtilis* α-amylases. The *amyR* linked tunicamycin resistant mutant which causes a five-fold increase in *B. subtilis* α-amylase, has no effect on the level of expression of the *amyV2* locus. Extra-cellular protease, alkaline phosphatase, and phosphodiesterase do not show altered thermostability, and the transformant is mesophilic in growth properties.

Lindsay and Creaser (*115*) showed that DNA from *B. caldolyticus* can transform *B. subtilis* for the ability to grow at 70°C. Surprisingly, they showed that the histidinol dehydrogenase (HDH) activity of the recipient is much more thermophilic than the *B. subtilis* HDH. They suggest that the genes responsible for conferring the ability to grow at high temperature alter the translation machinery of

the cell so that proteins produced from *B. subtilis* genes become more heat resistant. If this surprising idea were true, it would suggest a direct approach for making α-amylases more heat resistant (i.e., one might be able to transform a mesophilic α-amylase producer with DNA from an extreme thermophile and obtain a thermophilic α-amylase.)

Friedman and Mojica-a (*116*) transformed *Bacillus subtilis* with DNA from *B. caldolyticus* and *B. stearothermophilus*, selecting for ability to grow at elevated temperature (65°C). An examination on two-dimensional gels of ribosomal proteins from these rare transformants showed that they were identical to those of the strain donating the DNA; revertants restricted to growth at low temperature had ribosomal proteins like that of the *Bacillus subtilis* recipient. In this case, there was no report of enhanced stability of other cellular enzymes.

Summary

1. Both *Bacillus licheniformis* and *Bacillus stearothermophilus* produce an extremely thermostable α-amylase with a low requirement for Ca^{+}. The *B. licheniformis* enzyme has a broad pH range for activity and the *B. stearothermophilus* enzyme operates and is stable at somewhat lower pH.

2. Classical mutation and selection and combination of mutants in a single strain through transformation have resulted in *Bacillus subtilis* strains showing very high production of α-amylase.

3. *B. subtilis* transformed with a pUB110/*B. amyloliquefaciens* α-amylase chimeric plasmid is also a very prolific producer of the enzyme.

4. It may be possible to combine classical and recombinant DNA methodology to produce extremely high levels of a very thermostable/thermoactive enzyme.

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