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Review

Thermostable enzymes for industrial applications

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SUMMARY

The variety of thermostable (TS) enzymes has been steadily increasing for use in industrial applications, mainly as replacements for thermolabile (TL) enzymes. For example, TS amylases from *Bacillus licheniformis* and *Bacillus stearothermophilus* have replaced TL amylases from *Bacillus subtilis*. TS enzymes also have advantages in new areas such as cyclodextrin production. The TS cyclodextrin glycosyl transferase (CGTase) from *Thermoanaerobacter* sp. (95 °C optimum) gives a higher productivity than the CGTase from *Bacillus macerans* (55 °C optimum). In the area of enzymatic bleach boosting of wood pulps, a TS xylanase (*Myceliopthera thermophila*) would be advantageous over a TL xylanase (*Trichoderma reesei*), due to the high temperature of the incoming pulp. Not all TS enzymes are from thermophiles; the mesophile *Candida antarctica* produces a TS lipase which has a temperature optimum of 90 °C when immobilized. The characterization of these enzymes will be described along with comparisons to some newly described TS enzymes.

INTRODUCTION

The discovery of a variety of new and more active enzymes has led to expanded growth in the industrial enzyme market. Many of the newly developed enzymes have enhanced thermostability as compared to previously available enzymes. Interest in thermostable enzymes has grown, mainly due to the fact that most of the existing industrial enzyme processes are run at high temperatures using enzymes from mesophilic sources, e.g., high fructose corn syrup manufacturing.

There are quite a few advantages in using thermostable (TS) enzymes in industrial processes as compared to thermolabile (TL) enzymes [27]. The main advantage is that as the temperature of the process is increased, the rate of reaction increases. A 10 °C increase in temperature approximately doubles the reaction rate, which in turn decreases the amount of enzyme needed [59]. The TS enzymes are also able to tolerate higher temperatures, which gives a longer half-life to the enzyme. This is useful in systems such as glucose isomerase, which is used at high temperatures (50–65 °C) in immobilized reactors for periods up to 12 months. The use of higher temperatures (above 60 °C) also is inhibitory to microbial growth, decreasing the possibility of microbial contamination.

The use of high temperatures in industrial enzyme processes may also be useful in mixing, causing a decrease in the viscosity of liquids and may allow for higher concentrations of low solubility materials. The mass transfer rate is also increased at higher temperatures as is the rate of many chemical reactions.

The use of more highly thermostable enzymes from thermophilic organisms has been increasing, partly due to the ability to clone the genes from the thermophiles into mesophilic production strains [3,6]. For example, *Bacillus stearothermophilus* genes coding for thermostable α amylase and thermostable neutral protease have been cloned and expressed in *Escherichia coli* and *B. subtilis* [3,16,44].

The main application for thermostable enzymes has predominantly been starch liquefaction using amylases from *B. licheniformis* and *B. stearothermophilus* and proteases for food processing and detergents. Recently, some new areas for enzyme applications have been developed where the use of TS enzymes would be advantageous. These new areas are the production of cyclodextrins using cyclodextrin glycosyl transferase (CGTase) and biobleaching of wood pulps using xylanases.

This paper describes the characterization of some new TS enzymes for use in food processing, lipid modification, cyclodextrin production, and biobleaching of wood pulps.

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Comparisons of these TS enzymes with existing industrial enzymes and some newly described enzymes in literature will also be described.

PROTEASES

Proteases are the most widely used class of enzymes available. Sales of proteases have reached upward of U.S. $230 million in 1981 [9]. By far the greatest use of proteases is for laundry detergents, where they are used to remove protein-based stains. The second largest application of proteases involves the rennins, either microbial or bovine, for cheese-making. Other uses for proteases are in the baking, brewing and food industry (enzyme modified soy protein, meat tenderizers) and for leather softening.

There is a wide variety of proteases described in literature and available commercially. Proteases fall into groups dependent on whether they are active under acidic, neutral, or alkaline conditions and on characteristics of the active site group of the enzyme i.e., metallo-, sulphydryl or serine [58]. Proteases are also greatly influenced by temperature and a wide variety of thermostable proteases has been described.

While many proteases from thermophilic organisms have been studied, the most effort has been concerned with thermolysin, produced by *Bacillus thermoproteolyticus* [13]. Thermolysin is a neutral metalloprotease that requires zinc for activity and calcium for thermostability [29,61]. Recently, another commercial enzyme (SP 369, Novo Nordisk A/S) with characteristics similar to thermolysin has become available. This enzyme (SP 369) is produced by an asporogenous mutant of *B. stearothermophilus* and has a temperature optimum of 80 °C at pH 7.0 [61]. These enzymes are both useful in the hydrolysis of proteins at high temperatures and the enzymatic production of aspartame and other peptides [37,39].

A comparison of the temperature optima of the thermostable neutral protease, SP 369, with a neutral metalloprotease (Neutrase®, Novo Nordisk A/S) produced by *B. subtilis* is shown in Fig. 1. Neutrase has a temperature optimum of 45 °C, while SP369 has an optimum of 80 °C (in the presence of 10 mM $CaCl_2$).

The thermostability of SP 369 is also much greater than that of Neutrase. After 1 h incubation at 60 °C, Neutrase only maintains 20% of its original activity, while SP 369 maintains 90% of its original activity after 1 h at 80 °C and maintains approximately 60% of its original activity after one hour at 85 °C (Fig. 2).

In the enzymatic production of highly hydrolyzed proteins such as casamino acids, the hydrolysis must be carried out for extended periods (24–48 h). Hydrolysis with enzymes such as Neutrase and pancreatin must be performed at temperatures between 40 and 50 °C. Hydrolysis at these temperatures means that a bacteriostat such as ethanol or toluene must be added to avoid microbial contamination. These bacteriostats must be removed at considerable cost before the final product can be marketed. The use of a thermostable protease would be advantageous in this process.

A comparison of protein hydrolysis using Neutrase at 50 °C and SP 369 at 70 °C was performed in a CSTR. The substrate concentrations were 5% w/v and the enzymes were dosed at approximately equal enzymatic activity. The calcium concentration was 1.4 mM except where noted, and the pH was controlled at 7.0 by the addition of 1 N NaOH. The hydrolysis was monitored by determining the level of free amino nitrogen (FAN) generated by the enzymes.

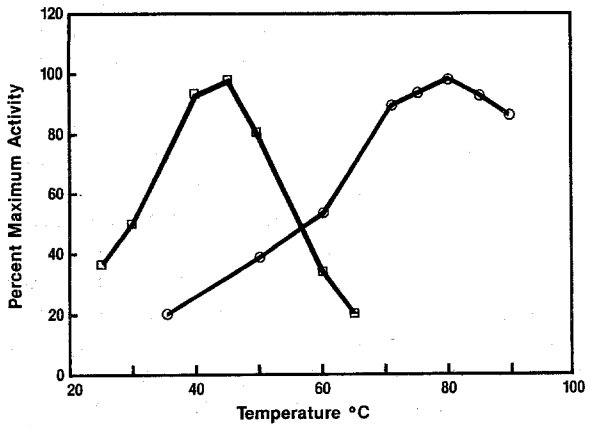

Fig. 1. Temperature profile of neutral proteases Neutrase and SP 369 at pH 7.0 □, Neutrase; ○, SP 369.

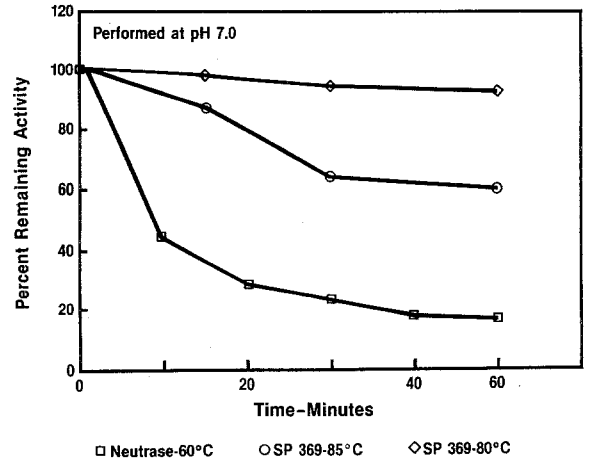

Fig. 2. Comparison of thermostabilities of Neutrase and SP 369 at pH 7.0 □, Neutrase, 60 °C; ◇, SP 369, 80 °C; ○, SP 369, 85 °C.

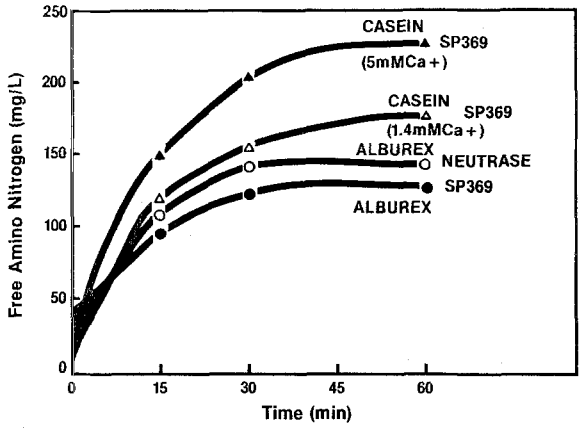

Fig. 3. Comparison of protein hydrolysis with Neutrase (50 °C) and SP 369 (70 °C). Substrate concentrations were 5% w/v and the calcium concentration was 1.4 mM except where noted.

The results, shown in Fig. 3, indicate that SP 369 can give similar hydrolysis rates at 70 °C as compared to Neutrase at 50 °C. It appears that the calcium concentration used (1.4 mM) was not adequate for hydrolysis with SP 369. Increasing the calcium level to 5 mM gave higher rates of casein hydrolysis.

Thermolysin and SP 369 both require calcium for thermostability and the level of thermostability is dependent on the level of calcium present [61]. A comparison of thermostable proteases from a variety of *Bacilli* and the effects of calcium on thermostability is shown in Table 1. Calcium appears to be required by all the enzymes listed, regardless of whether they are a metallo- or serine protease. It is interesting to note that the serine protease from *B. thuringiensis* is very thermostable, yet the strain is a mesophile.

The thermostability (half-lives) and characteristics of some other proteases are detailed in Table 2. The proteases aqualysin and caldolysin, produced by strains of *Thermus aquaticus*, are very thermostable [7,33]. Caldolysin has a half-life of over 190 h at 75 °C (10 mM $CaCl_2$) and aqualysin has a half-life of 3 h at 80 °C without calcium. The alkaline proteases from *Streptomyces* also need calcium for thermostability.

The protease pyrolysin, from *Pyrococcus furiosis*, has the greatest thermostability of any reported protease, showing a half-life of 3600 min at 98 °C [47]. The protease thermopsin is an unusual acid protease, having a pH optimum of 2.0 and a temperature optimum of 90 °C. This enzyme shows no loss of activity after 48 h at 80 °C [17,30].

Some of the more interesting proteases are produced by archaebacteria. *Desulfurococcus* sp. produces a serine protease, archaelysin, with a half-life of 9 min at 105 °C [8], while the protease from *Thermococcus celer* has a half-life of 40 min at 95 °C [6].

XYLANASES

The use of xylanases to break down hemicellulosic materials has been extensively studied. Xylanases are secreted by a variety of bacteria, yeast, and fungi. They are composed of mixtures of endo-xylanases (EC 3.2.1.8) and

TABLE 1

Comparison of thermostable proteases from various strains of *Bacilli* and the effect of calcium on thermostability

Strain	Enzyme class	Optimum		Thermostability	$Cacl_2$ (mM)	Ref.
		pH	Temp.			
B. stearothermophilus	M	7.0	80 °C	7%/60 min/85 °C	2	[61]
				60%/60 min/85 °C	10	
B. thermoproteolyticus	M	7.0	80 °C	7%/60 min/85 °C	2	[61]
				60%/60 min/85 °C	10	
B. thermoruber	S	9.0	45 °C	40%/60 min/60 °C	0	[31]
				100%/60 min/60 °C	2	
B. sp. AH-101	S	12.0	80 °C	50%/10 min/70 °C	0	[50]
				60%/10 min/80 °C	5	
B. thuringiensis	SH-S	9.0	70 °C	50%/30 min/60 °C	0	[28]
				88%/420 min/60 °C	2	
B. cereus	M	7.0	50 °C	90%/40 min/75 °C	10	[46]

Thermostability is expressed as percent remaining activity after incubation at specified time (min) and temperature.
Enzyme class: M = metalloprotease, S = serine protease, SH-S = sulfhydryl-serine protease.

TABLE 2

Thermostability (half-lives) and characteristics of various thermostable proteases

Strain	Enzyme class	optimum		$t_{1/2}$	Ref.
		pH	Temp.		
Bacillus licheniformis Subtilisin A 1	S	9.0	60 °C	50 min/65 °C	[24,62]
B. thermoproteolyticus Thermolysin	M	7.0	80 °C	15 min/85 °C	[13,61]
Thermus aquaticus YT-1 Aqualysin I	S	10.0	75 °C	180 min/80 °C	[33]
Thermus aquaticus T351 Caldolysin	M	8.0	90 °C	60 min/90 °C >10000 min/75 °C	[7]
Streptomyces sp. 1689 Protease A	SH-S	10.5	80 °C	30 min/80 °C	[35]
Streptomyces rectus var. *proteolyticus*	S	9.0	70 °C	30 min/80 °C	[9,35]
Desulfurococcus sp. Archaelysin	S	8.0	100 °C	90 min/95 °C 9 min/105 °C	[8]
Thermomonospora fusca YX Protease	S	9.0	80 °C	15 min/85 °C	[21]
Pyrococcus furiosis Pyrolysin	ND	6.8	98 °C	3600 min/98 °C	[47]
Sulfolobus acidocaldarius Thermopsin	ND	2.0	90 °C	>90 min/90 °C[a]	[17,30]
Vibrio proteolyticus Vibriolysin	ND	8.0	65 °C	20 min/65 °C	[11]

[a] Enzyme shows no loss of activity after 90 min.
ND, not described.

exo-xylanases, such as β-D-xylosidase (EC 3.2.1.37). There are currently four major applications for xylanases; (1) enzymatic breakdown of agricultural wastes for production of alcohol fuels; (2) enzymatic treatment of animal feed to release free pentose sugars; (3) manufacturing of dissolving pulps yielding cellulose for rayon production; and (4) bio-bleaching of wood pulps [4,10,40,42].

The pulp and paper industry is using xylanase compositions in the bio-bleaching process to enhance the brightness of bleached pulps, to decrease the amount of chlorine used in the bleaching stages, and to increase the freeness of pulps in the recycled paper process [14,40,41,45,54].

Kraft pulping, a process widely used in the pulp and paper industry, involves the alkaline cooking of pulp to remove 95% of the lignin present in wood. The remaining 5% of the lignin gives the pulp a dark brown color, which has the tendency to darken in UV light or by oxidation. In order to obtain a white pulp for high quality paper, the brown color is removed by a multi-stage bleaching process using chlorine or chlorine dioxide.

Presently there is much concern about the environmental impact of the chemicals generated from the bleaching process. Enzymes can aid in the removal of lignin from the pulp without any harmful side products. Reports show that lignin in wood is linked to xylan, possibly through an arabinose side chain [15,51]. By hydrolyzing the xylose-xylose bonds (xylanase, xylosidase) and arabinose xylose bonds (xylanase, arabinase), a greater release of lignin occurs during bleaching. Thus, by enzymatically treating the pulp prior to bleaching, the amount of active chlorine needed would decrease.

Recently the first commercially available xylanase for use in the bio-bleaching of wood pulps has been introduced. The enzyme, Pulpzyme™ HA (Novo Nordisk A/S) is a hemicellulase containing enzyme preparation produced by a strain of *Trichoderma reesei* and is capable of decreasing the dosage of active chlorine in the first bleaching stage [41].

Studies were performed with Pulpzyme HA on oxygen delignified birch kraft pulp to find an effective enzyme dose. Fig. 4 shows the effect of enzyme dosage on the Kappa number of enzyme treated pulp. The Kappa number is an indication of the amount of lignin present. The

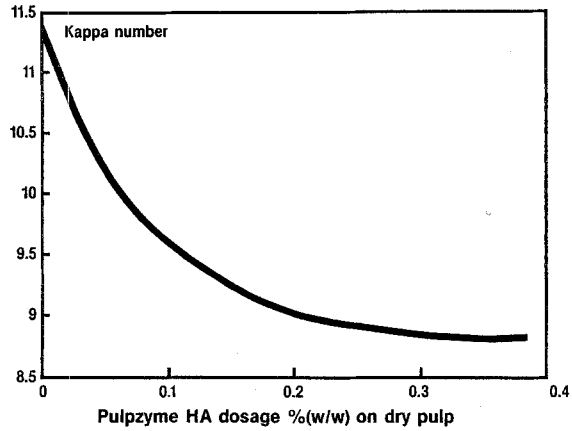

Fig. 4. Effect of Pulpzyme HA dosage on the Kappa number of an oxygen delignified birch kraft pulp.

Kappa number was reduced approximately 20% by using a Pulpzyme HA dose of 0.15% (w/w). The enzyme was added to the pulp (10% consistency) and the mixture was incubated in plastic bags for 3 h at 50 °C, pH 6.0.

The use of Pulpzyme HA in an industrial setting presents a few problems. First, the temperature of the incoming pulp is around 70 °C, which is above the range for the enzyme. Secondly, the pH of the pulp is in the alkaline range, requiring pH adjustment to around 7.0. Also the treatment time of 3 h may also contribute to inactivation of the enzyme, especially if the pulp is too hot, and may be too long to be practical. These problems might be overcome by the use of a thermostable xylanase.

The xylanase temperature activity profile for Pulpzyme HA was compared to the xylanase from *Myceliopthera thermophila*, a strain of thermophilic fungi shown to produce an extremely thermostable cellulase complex [43]. The results comparing the *Trichoderma reesei* xylanase to the *M. thermophila* (CBS 117.65) xylanase (Fig. 5) show the *T. reesei* xylanase to have a temperature optimum of 50 °C, and the xylanase from *M. thermophila* to have an optimum of 70 °C, a temperature where the *T. reesei* xylanase is inactive.

The thermostabilities of the two xylanases, compared at pH 7.0, are shown in Fig. 6. The *T. reesei* xylanase maintains approximately 70% activity after 3 h at 50 °C, but is not very stable at 55 °C, losing 70% activity after 30 min. The *M. thermophila* xylanase is much more thermostable, showing no loss of activity after 3 h at 50 °C and showing a loss of 50% after 2 h at 60 °C. Both enzymes were compared in buffer only, and the enzymes may show greater thermostability in the presence of the pulp.

The characteristics of other thermostable xylanases produced by thermophilic fungi and bacteria are shown in Table 3. The xylanase from the thermophilic fungus *Talaromyces byssochlamydoides* has a temperature optimum of 75 °C, and shows good thermostability, retaining 65% activity after five minutes at 95 °C [55]. The xylanases from the *Thermomonospora* strains are also very stable maintaining over 90% activity after 1 h at 70 °C [34]. Xylanase A from the thermophilic anaerobe *Clostridium stercorarium* has a temperature optimum of 75 °C and a half-life of 90 min at 80 °C [2], while the xylanase from *Thermotoga* sp. has a half life of over 20 min at 105 °C [6].

LIPASES

Several reactions are catalyzed by lipases: (1) hydrolysis of lipids; (2) acidolysis (replacement of a esterified

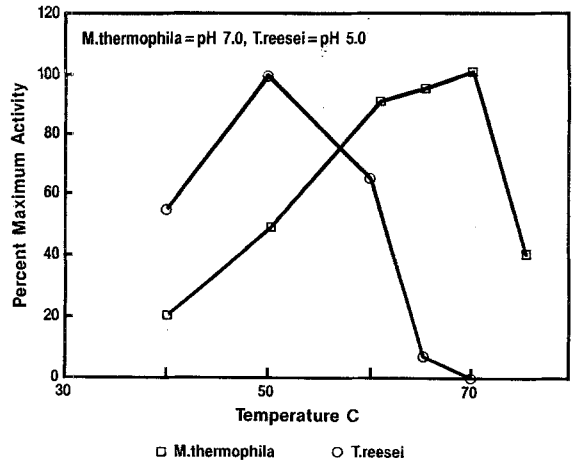

Fig. 5. Temperature profiles of xylanases from *Myceliopthera thermophila* and *Trichoderma reesei* (Pulpzyme HA). □, *M. thermophila*; ○, *T. reesei.*

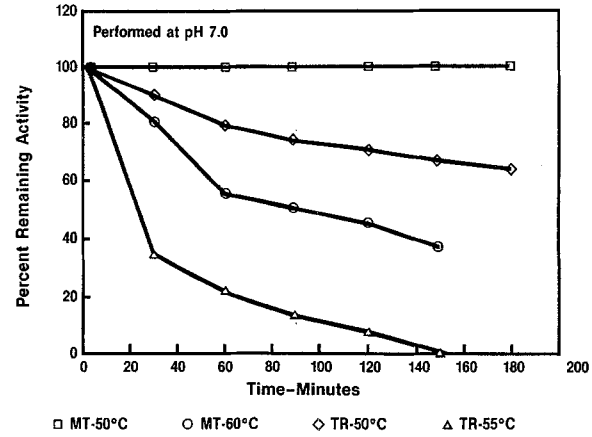

Fig. 6. Comparison of thermostabilities of the xylanases from *M. thermophila* and *T. reesei.* Comparisons were performed in buffer at pH 7.0. □, *M. thermophila*, 50 °C; ○, *M. thermophila*, 60 °C; ◇, *T. reesei*, 50 °C; △, *T. reesei*, 55 °C.

TABLE 3

Characteristics of thermostable xylanases

Strain	Optimum		Thermostability	Ref.
	pH	Temp.		
Thermophilic fungi				
Paecilomyces variota	6.5	65 °C	65%/60 min/70 °C	[26]
Malbranchea pulchella var. *sulfurea*	6.5	70 °C	100%/30 min/60 °C	[32]
Talaromyces byssochlamydoides	5.0	75 °C	65%/5 min/95 °C	[55]
Thermoascus auranticus	5.0	75 °C	50%/90 min/70 °C	[60]
Chaetomium thermophile var. *coprophile*	6.0	70 °C	50%/60 min/60 °C	[18]
Myceliopthera thermophila	6.5	70 °C	50%/120 min/60 °C	This paper
Thermophilic bacteria				
Thermomonospora fusca	6–8	70 °C	90%/60 min/70 °C	[34]
Thermomonospora chromogena	6.0	75 °C	98%/60 min/70 °C	[34]
Bacillus acidocaldarius	4.0	80 °C	90%/15 min/75 °C	[56,57]
Thermophilic *Bacillus* sp.	7.0	78 °C	60%/420 min/74 °C	[20]
Alkalophilic *Bacillus* sp.	7.0	65 °C	45%/60 min/70 °C	[23]
Clostridium stercorarium Xylanase A	6.5	75 °C	50%/90 min/80 °C	[2]

Thermostability is expressed as percent remaining activity after incubation at specified time (min) and temperature.

fatty acid with a free fatty acid); (3) transesterification (exchange of fatty acids between triglycerides); and (4) ester synthesis [22]. Most of these reactions are preferably done at low water activity to prevent hydrolysis of the desired ester [12]. In order to avoid the use of solvents, lipase reactions are often carried out in mixtures of the reactants. For this to be feasible, the reaction mixture has to be heated from 50 °C to 80 °C for the fat to be liquid. Lipases therefore have to be thermostable for optimal performance.

Some lipases show a high degree of specificity for the outer 1 and 3 positions of triglycerides; for example, *Mucor miehei* lipase (Lipozyme™), and a *Humicola* lipase expressed in *Aspergillus oryzae* (Lipolase™). These lipases are used for specific modifications of triglycerides or in laundry detergents [12,19]. Other lipases are non-specific; for example, the lipases of *Candida cylindracea* and *C. curvata*. The non-specific lipases are advantageous for total hydrolysis, some interesterifications and for ester synthesis [22,36,53].

Recently a very thermostable non-specific lipase has been discovered from *Candida antarctica* [22]. *C. antarctica* is a mesophilic fungi with an optimum growth temperature of 26–30 °C; it produces at least two lipases with component A being the most thermostable. Fig. 7 compares the thermoactivity of this lipase with two other fungal lipases: The *C. antarctica* lipase A has a temperature optimum at or above 70 °C whereas the two other

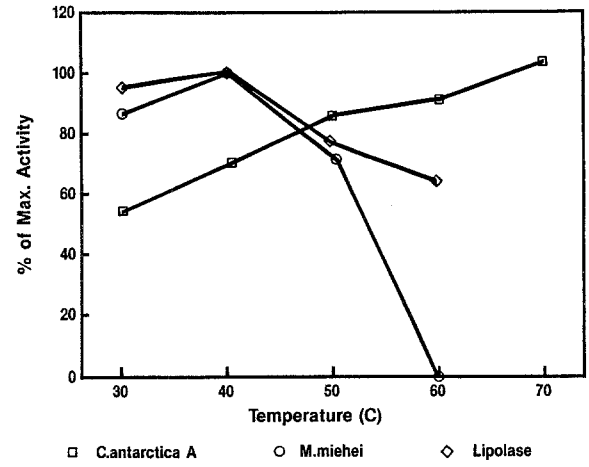

Fig. 7. Temperature profile of three fungal lipases. □, *Candida antarctica*; ○, *Mucor miehei*; ◇, Lipolase.

lipases have optima around 40 °C. Using different assays, Kimura et al. [25] found the *C. cylindracea* lipase to have a temperature optimum at 30 °C, and Montet et al. [36] found the *C. curvata* lipase to have an optimum between 50 and 60 °C. Additionally, Table 4 shows that the *C. antarctica* lipase also is more thermostable than other lipases.

Industrially, lipases used for transesterification, acidolysis or ester synthesis are immobilized to increase their thermostability and extended use in columns. Fig. 8

TABLE 4

Thermostability of four soluble fungal lipases

Strain	Temperature optimum	Thermostability	Ref.
Candida antarctica A	70 °C	98% (pH 6.5)	[22]
Candida cylindracea	30 °C	10% (pH 6.5)	[25]
Candida curvata CBS 570	50–60 °C	1% (pH 6.5)	[36]
Mucor miehei	40 °C	1% (pH 7.0)	This paper

Thermostability is expressed as percent remaining activity after 1 h incubation at 60 °C and at the indicated pH.

shows that the temperature optimum of the *M. miehei* lipase increased to about 70 °C after immobilization. Kimura et al. [25] showed similarly that the temperature optimum of the *C. cylindracea* increased to about 40 °C.

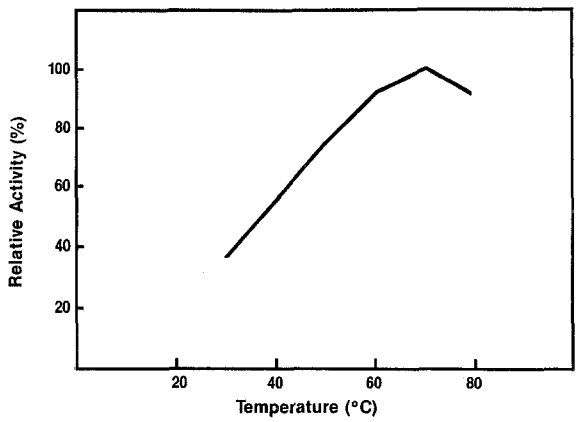

Fig. 8. Temperature profile of immobilized *Mucor miehei* lipase.

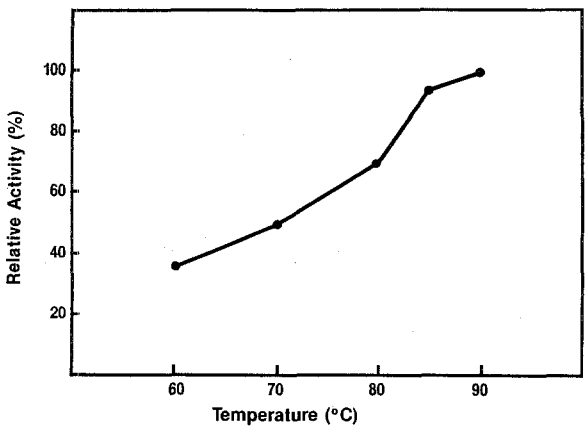

Fig. 9. Temperature profile of immobilized *Candida antarctica* lipase.

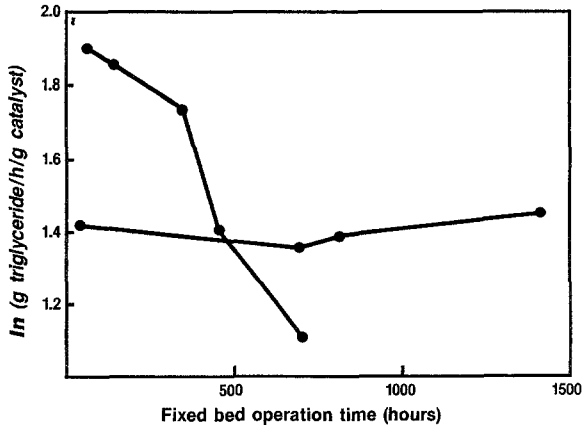

Fig. 10. Continuous interesterification in a fixed bed reactor with immobilized *Candida antarctica* lipase. The substrate contained 70% soy bean oil and 30% (w/w) lauric acid (water saturated). The reactions were performed at 60 °C and 80 °C.

After immobilization of the crude *C. antarctica* lipase, a temperature optimum of about 90 °C was observed (Fig. 9) [22].

The immobilized *C. antarctica* lipase has been found to be effective in a number of applications such as synthesis of esters using secondary alcohols [22], for synthesis of glycolipids [5] and for acidolysis (Fig. 10). When incorporating lauric acid into soybean oil, the enzyme was found to have a half-life at 80 °C of about 700 h; whereas at 60 °C, no decrease in activity was observed after 1400 h.

CYCLODEXTRIN GLYCOSYL TRANSFERASES

Starch can be degraded by a group of enzymes known as cyclodextrin glycosyl transferases (CGTase, 1,4-α-D-glucan-4-α-D-(1,4-α-D-glucano)-transferase, EC 2.4.1.19). The CGTase enzymes degrade starch by catalyzing cyclization and disproportionation reactions as shown below [1]:

$$\text{(1)}\ \text{glc}_n \xrightleftharpoons[\text{coupling}]{\text{cyclization}} \text{glc}\,(n-x) + x$$

$x = 6$ α-cyclodextrin
$x = 7$ β-cyclodextrin
$x = 8$ γ-cyclodextrin

$$\text{(2)}\ \text{glc}_m + \text{glc}_n \xrightleftharpoons{\text{disproportionation}} \text{glc}\,(m+x) + \text{glc}\,(n-x)$$

The cyclization reactions produce cyclodextrins (also known as Schardinger dextrins) which are cyclic molecules comprised of six, seven, or eight α-D-glucopyranose residues linked by α-1,4 bonds, and are known as α-, β-, or γ-cyclodextrins respectively. These cyclic molecules have neither a non-reducing nor reducing end-group.

The production of cyclodextrins is generally accomplished by variations of the methodology described by Tilden and Hudson, involving the CGTase from *Bacillus mascerans* [52]. A major disadvantage of the method is that the starch must be solubilized by a pretreatment with an α-amylase. Following liquefaction, the α-amylase must then be inactivated in order to obtain good cyclodextrin yields. Another disadvantage is that the *B. mascerans* CGTase is not sufficiently thermostable to be used at elevated temperatures. Consequently, conversion of starch to cyclodextrins requires an extended reaction time before reasonable yields are achieved. Further, microbial contamination can become a significant problem.

Other producers of CGTase include *B. circulans*, *B. stearothermophilus*, *B. megaterium*, *B. licheniformis*, *B. ohbensis*, alkalophilic *Bacillus* sp., *Micrococcus varians*, and *Klebsiella pneumoniae* [1]. However, none of these CGTases are sufficiently more stable than the *B. mascerans* enzyme to eliminate these disadvantages.

Integration of standard industrial enzymatic starch liquefaction [38] with the enzymatic conversion of liquefied starch into cyclodextrins would be most advantageous. Since CGTases can degrade starch by catalyzing cyclization and disproportionation reactions, a CGTase characterized by a high thermostability, similar to the α-amylases employed by the starch industry, should be able to solubilize the starch, thereby eliminating the need for α-amylase pretreatment. Further, the high thermostability should shorten the cyclodextrin production time.

A group of thermophilic, anaerobic microorganisms belonging to the genus *Thermoanaerobacter* have been discovered to be capable of producing highly thermostable CGTases. The ability of one of these enzymes from *Thermoanaerobacter* sp. ATCC 53627 to liquefy starch [48] and to produce cyclodextrins [49] has been established.

The *Thermoanaerobacter* sp. CGTase preparation has pH and temperature optima for activity at pH 5.0 and 95 °C (pH 5.0). The enzyme retains 95–100% activity in the absence of substrate and calcium at 80 °C, pH 5, for 40 min. This CGTase preparation is the most thermostable reported to date.

The thermostability of this enzyme allows it to be used to liquefy starch. Industrially, heating of the starch during primary liquefaction is accomplished by jet-cooking. When a 35% DS corn starch slurry is liquefied with the CGTase at pH 4.5 by jetting at 105 °C for 5 min (primary liquefaction) followed by a hold at 90 °C (secondary liquefaction), a rapid decrease in viscosity is observed as shown in Fig. 11. With thermolabile CGTases, the starch has to be preliquefied leading to extra costs and potentially lower yield [52].

A comparison with *B. mascerans* CGTase shows that at 50 °C, the *Thermoanaerobacter* sp. CGTase is superior

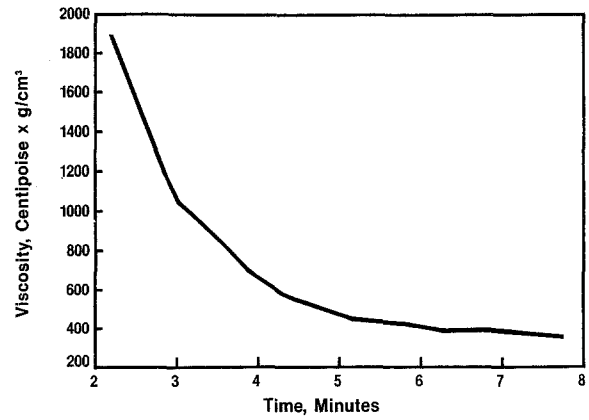

Fig. 11. Viscosity reduction of a corn starch slurry (35% DS) during secondary liquifaction with *Thermoanaerobacter* CGTase.

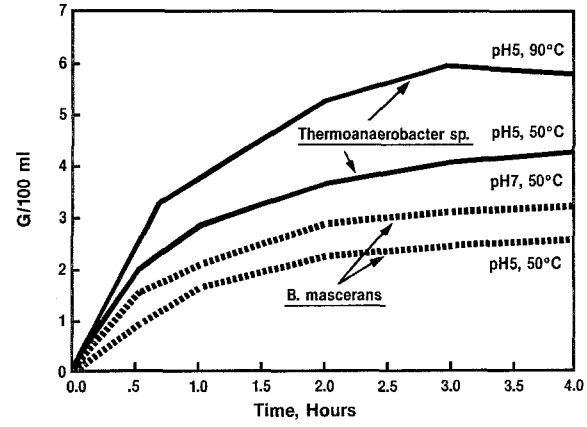

Fig. 12. Comparison of *Bacillus macerans* and *Thermoanaerobacter* CGTase for cyclodextrin production.

in cyclodextrin production from 15% DS Lintner starch in 4 hours (Fig. 12). Increasing the temperature to 90 °C essentially doubles the yield relative to that achieved with the *B. mascerans* CGTase at 50 °C. The cyclodextrin ratios are as follows ($\alpha/\beta/\gamma$): *Thermoanaerobacter* sp. CGTase: pH 5, 50 °C: 1.00/1.55/0.53; pH 5, 90 °C: 1.00/1.36/0.47. *B. mascerans* CGTase: pH 5, 50 °C: 1.00/0.76/0.33; pH 7, 50 °C: 1.00/1.06/0.37. The *thermoanaerobacter* CGTase provides several advantages for the production of cyclodextrins. Other CGTases are unable to liquefy starch at high temperatures and, therefore, the starch must be partially hydrolyzed with an α-amylase prior to CGTase addition. This adds cost and lowers the yield. The reaction time with the *Thermoanaerobacter* CGTase is shorter than if a thermolabile CGTase is used; typically 3–6 h vs. 1–3 days. Also, the higher temperature prevents microbial contamination, which can be a problem at lower temperatures. The *Thermoanaerobacter* CGTase, therefore, provides several significant advantages over currently used enzymes enabling more efficient production of cyclodextrins, and hopefully, expanded use.

The thermostability of the CGTase and its ability to liquefy starch is also an advantage in the production of high fructose corn syrup: enzymatic liquefaction of starch is generally accomplished at a pH of approximately 6.0–6.5, followed by a saccharification step performed at a pH of approximately 4.5.

A corn starch slurry (35% DS) was liquefied with the CGTase at pH 4.5 at 105 °C for 5 min followed by 2 h secondary liquefaction at 90 °C. The slurry was then cooled to 60 °C and Dextrozyme^{R} added and incubated for 48 h. The resulting syrup contained 95.4% glucose. This glucose yield is similar to those obtained commercially using α-amylases to liquefy the starch.

Lowering the liquefaction pH to 4.5 has several advantages: the starch from the wet-milling process has a pH of 4–5; thus little pH adjustment is needed prior to liquefaction. The following saccharification is typically run around pH 4.5; again the pH 4.5 liquefaction reduces the need for pH adjustments. Less pH adjustment leads to savings because of less acid and base needed for the adjustments. More importantly, however, the lower level of salts generated by the pH adjustments results in reduced need for ion exchange and chemicals in the purification of the syrup. Also, a lower pH during liquefaction reduces base-catalyzed by-product formation, which in turn leads to charcoal savings and potentially higher yields.

CONCLUSION

The use of thermostable enzymes in industrial processes allows reactions to be performed at higher temperatures and for shorter times, decreasing the possibilities of microbial contamination. The use of high process temperatures also allows for the use of reactants that would be insoluble at lower temperatures, i.e., fats. The commercial availability of thermostable enzymes has been increasing mainly to replace previously utilized, more thermolabile enzymes. The developments of new enzyme application markets such as bio-bleaching and cyclodextrin production should increase the future demand for thermostable enzymes.

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