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Recombinant Expression and Purification of a Thermostable Amylase for Industrial Starch Processing

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ABSTRACT

Background: Thermostable α-amylases from bacteria and archaea are central to industrial starch liquefaction, yet native producers rarely yield enough enzyme for commercial use. Objective: This review synthesises published evidence on recombinant expression, purification, and engineering of thermostable amylases. Methods: A narrative review of peer-reviewed studies on Bacillus, Geobacillus and Pyrococcus amylases expressed in Escherichia coli, Bacillus subtilis and Pichia pastoris. Findings: Host choice, signal-peptide selection, and rational or computational mutagenesis substantially raise yield, thermostability and specific activity. Conclusions: Combining robust hosts with structure-guided engineering remains the most reliable route to industrially viable thermostable amylases.

Keywords: *thermostable amylase; recombinant expression; Bacillus licheniformis; Pichia pastoris; starch liquefaction*

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Abstract

Background: Thermostable α-amylases from bacteria and archaea are central to industrial starch liquefaction, yet native producers rarely yield enough enzyme for commercial use. **Objective:** This review synthesises published evidence on recombinant expression, purification, and engineering of thermostable amylases. **Methods:** A narrative review of peer-reviewed studies on *Bacillus*, *Geobacillus* and *Pyrococcus* amylases expressed in *Escherichia coli*, *Bacillus subtilis* and *Pichia pastoris*. **Findings:** Host choice, signal-peptide selection, and rational or computational mutagenesis substantially raise yield, thermostability and specific activity. **Conclusions:** Combining robust hosts with structure-guided engineering remains the most reliable route to industrially viable thermostable amylases.

Keywords: thermostable amylase; recombinant expression; Bacillus licheniformis; Pichia pastoris; starch liquefaction

Introduction

Amylases (EC 3.2.1.1) hydrolyse the α-1,4-glycosidic bonds of starch and account for a substantial share of global industrial enzyme sales, with applications spanning detergents, textiles, brewing, baking, and biofuel production [1,2]. Among industrial processes, starch liquefaction and saccharification place the most extreme demands on enzyme stability: cornstarch slurries are gelatinised and liquefied at 90-110°C, conditions under which mesophilic amylases denature within minutes [3]. This has driven decades of interest in thermostable amylases from thermophilic and hyperthermophilic bacteria and archaea, including *Bacillus licheniformis*, *Geobacillus stearothermophilus*, and *Pyrococcus furiosus* [4].

Native production of these enzymes from their source organisms is typically slow, low-yielding, or logistically difficult, particularly for archaeal hyperthermophiles that grow poorly under standard laboratory conditions [5]. Recombinant expression in tractable heterologous hosts—chiefly *Escherichia coli*, *Bacillus subtilis*, and the methylotrophic yeast *Pichia pastoris*—has therefore become the standard route to industrially relevant quantities of thermostable amylase, often coupled with protein engineering to further enhance thermal resistance, catalytic efficiency, or secretion [6,7]. This review does not report a new experiment; rather, it is a narrative synthesis of published literature describing (i) the diversity and structural basis of amylase thermostability, (ii) heterologous expression platforms and their trade-offs, (iii) engineering strategies used to further improve stability and activity, (iv) purification approaches exploited for these enzymes, and (v) their downstream application in industrial starch processing. Real reported values from the cited primary literature are used throughout to illustrate the

magnitude of the improvements achievable.

Sources and Diversity of Thermostable Amylases

The best-characterised thermostable α-amylase is BLA, secreted natively by *Bacillus licheniformis*, which retains substantial activity above 90°C in the presence of calcium and is stabilised by an extended loop in domain B and a network of buried ion pairs identified by crystallography and mutational scanning [8]. Systematic combination of stabilising substitutions extended BLA's operational stability window across a 50°C range, demonstrating that thermostability and low-temperature flexibility can be decoupled through rational substitution [9]. *Geobacillus stearothermophilus*, a related thermophilic Firmicute, produces amylases such as the SR74 variant, which shows maximal activity at 65°C with a half-life of roughly 88 minutes at 60°C, declining to about 40 minutes at 70°C [10]; separate work on a maltooligosaccharide-forming amylase from strain STB04 attributes much of this thermal resilience to a calcium-ion triad within the structure [11].

At the extreme end of the thermal spectrum, archaea contribute some of the most stable amylolytic enzymes known. The hyperthermophile *Pyrococcus furiosus* produces an extracellular amylase whose gene was cloned and expressed heterologously more than two decades ago [5], and a related amylopullulanase-type activity from the same organism, designated PFTA, shows an optimal temperature near 90°C while additionally hydrolysing cyclodextrins and pullulan, blurring the boundary between amylase and cyclodextrin-hydrolysing enzyme classes [12]. Even more extreme is a hyperthermostable amylase gene from the hydrothermal-vent bacterium *Thermotoga petrophila*, whose recombinant product, expressed in *E. coli*, remained active at 100°C [13]. Beyond microbial hosts, the PFTA-related *P. furiosus* α-amylase has even been expressed in transgenic *Nicotiana tabacum*, where it accumulates as functional aggregates that retain hyperthermostable activity, illustrating that plant molecular farming is a further, if less conventional, production route for this enzyme class [14]. Fungal thermostable enzymes complement this bacterial and archaeal diversity: a glucoamylase from *Bispora* sp. MEY-1 retains 98% of initial activity after one hour at 70°C, versus only about 10% for a conventional *Aspergillus niger* glucoamylase under the same conditions, and remains stable across an unusually broad pH range of 2.2-11.0 [15]. Figure 1, placed under this section, summarises reported optimal temperatures across these representative enzymes, illustrating the roughly 35°C spread between the least and most thermostable characterised amylases.

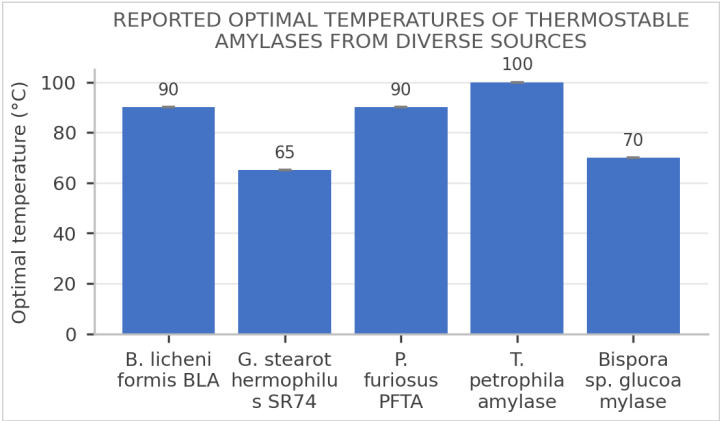
Recombinant Expression Systems for Thermostable Amylases

Escherichia coli remains the default host for initial cloning and functional characterisation of thermophilic and archaeal amylase genes because of its rapid growth, well-developed genetic toolkit, and compatibility with strong inducible promoters; the original *P. furiosus* amylase gene was expressed successfully in both *E. coli* and *B. subtilis* to confirm activity and establish biochemical parameters before further optimisation [5]. *B. subtilis* offers the added advantage of native extracellular secretion machinery closely related to that of thermophilic *Bacillus* relatives, and systematic screening of signal peptides—including the identification of the YoaW signal peptide, which improved secretion of heterologous fusions several-fold over conventional choices—has substantially increased achievable titres in this host [16]. Codon-optimised *E. coli* signal peptides have likewise been shown to influence periplasmic localisation, yield, and activity of a recombinant *Geobacillus stearothermophilus* maltogenic amylase, underscoring that secretion signal choice, not just promoter strength, governs recoverable enzyme [17].

Table 1: Representative recombinant host systems used for thermostable amylase expression and reported production outcomes.

Host	Example enzyme (source organism)	Reported outcome	Reference
Escherichia coli	Extracellular amylase, Pyrococcus furiosus	Functional heterologous expression enabling biochemical characterisation	[5]
Bacillus subtilis	Heterologous fusions via YoaW signal peptide	Several-fold higher secretion than conventional signal peptides	[16]
Pichia pastoris	Geobacillus stearothermophilus SR74 amylase	Secreted directly to supernatant; optimal activity at 65°C retained	[10]
Pichia pastoris	Bacillus licheniformis amylase (marker-free)	Production of approximately 900 U/mL, thermostability unaffected by glycosylation	[18]
Pichia pastoris (multi-copy, PTVA)	Chimeric acidic α-amylase	10.7-fold titre increase over single-copy transformant	[19]

Figure 1: Reported optimal temperatures of representative thermostable amylases from bacterial, archaeal and fungal sources, compiled from published characterisation studies [8,10,12,13,15].



Pichia pastoris has become particularly attractive for secreted, glycosylated thermostable amylases destined for industrial use, since it combines eukaryotic secretory processing with fermentation densities and yields approaching bacterial systems. Recombinant *G. stearothermophilus* SR74 amylase expressed in *P. pastoris* retained the thermal profile described above while being secreted directly into the culture supernatant, simplifying downstream recovery [10]. Similarly, *B. licheniformis* amylase expressed in *P. pastoris* without an antibiotic-resistance marker reached production levels of approximately 900 U/mL, and comparison with a non-glycosylated bacterial counterpart showed that yeast-derived glycosylation did not compromise

thermostability [18]. Multi-copy integration strategies further raise titres: a chimeric acidic α-amylase produced via post-transformational vector amplification in *P. pastoris* achieved a 10.7-fold increase in enzyme titre relative to a single-copy transformant, while retaining acid stability and thermostability suited to saccharification applications [19]. Fungal amylases have also been engineered into this host, as with *Aspergillus oryzae* AmyA, whose *P. pastoris*-expressed, N-glycosylated form showed a higher optimal temperature (60°C) than the native enzyme (50°C) [20]. Table 1, presented under this section, compares representative host systems, example enzymes, and reported production outcomes drawn from these studies.

Protein Engineering Strategies for Enhanced Thermostability

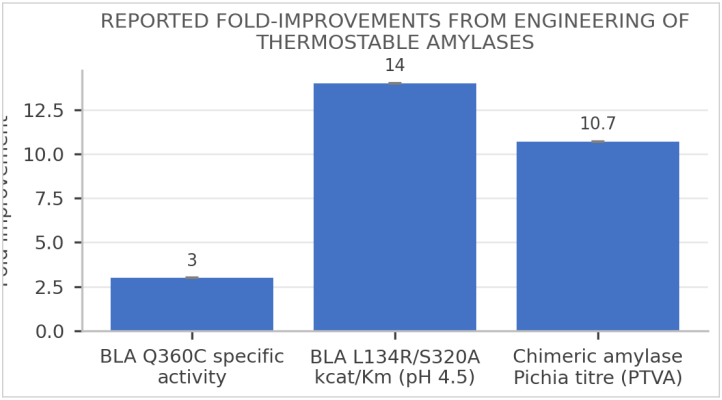
Beyond host selection, structure-guided and computational engineering has repeatedly delivered large, quantifiable gains. Rational redesign of BLA guided by sequence-consensus analysis and folding free-energy calculations produced a Q360C mutant with nearly threefold higher specific activity than wild type, alongside improved residual activity after heat challenge at 70°C [6]. A complementary computational approach identified additional stabilising substitutions in BLA that increased both catalytic performance and high-temperature stability [7]. Acid tolerance has been engineered similarly: introducing paired substitutions L134R and S320A into BLA raised the catalytic efficiency (kcat/Km) at pH 4.5 roughly 14-fold relative to the wild-type enzyme, reflecting altered electrostatics around the active site under acidic, industrially relevant conditions [21]. In *Geobacillus stearothermophilus* amylase, deep-learning-guided identification of residues along the enzyme's conformational motion pathway led to a P44E substitution that widened internal substrate channels and raised the optimal temperature to 90°C with improved catalytic efficiency [22]. Broader reviews of acid-stable amylases from acidophilic sources catalogue further families of mutations that jointly improve production, catalytic efficiency, thermostability, and starch-saccharification performance [23], while comprehensive surveys of microbial α-amylases more generally emphasise that mass-transfer limitations, low yields, and energy-intensive recovery remain the principal bottlenecks that engineering and bioprocess optimisation must jointly address [3]. Figure 2, under this section, summarises reported fold-improvements from three representative engineering studies.

Purification and Downstream Processing

Table 2: Purification strategies exploited for recombinant thermostable amylases.

Strategy	Principle	Illustrative system	Reference
Heat-precipitation of host protein	Exploits target enzyme's superior thermal stability relative to host proteome	Thermotoga petrophila amylase in E. coli lysate	[13]
Direct recovery from culture supernatant	Secretory host releases folded, active enzyme extracellularly	Geobacillus stearothermophilus amylase in Pichia pastoris	[10]
Marker-free secretory production	Antibiotic-resistance-free secretion simplifies downstream recovery	Bacillus licheniformis amylase in Pichia pastoris	[18]
One-step self-immobilisation	Fusion to polyester-granule protein PhaC yields reusable enzyme-coated beads	Thermostable amylase-PhaC fusion in E. coli	[24]

Figure 2: Fold-improvements in specific activity, catalytic efficiency, or expression titre reported for three engineering case studies on thermostable amylases [6,21,19].



Because target amylases are far more heat-tolerant than the bulk of host-cell protein, a brief thermal incubation step is commonly exploited as a simple, low-cost enrichment step before chromatographic polishing, a strategy consistent with the extreme stability reported for the *Thermotoga petrophila* amylase recovered from *E. coli* lysate [13]. Secretory hosts simplify purification further by releasing the folded enzyme directly into culture supernatant, avoiding cell lysis and the associated contaminant burden; this is illustrated by both the *Pichia pastoris*-secreted *G. stearothermophilus* amylase [10] and the antibiotic-marker-free *B. licheniformis* amylase system, which is recovered directly from spent medium at high titre [18]. An alternative to conventional chromatography is one-step self-immobilisation, achieved by fusing a thermostable amylase to the polyester-granule-forming protein PhaC in *E. coli*, yielding polymer beads that carry active, reusable enzyme without any separate immobilisation chemistry and that tolerate repeated catalytic cycles at 75-85°C [24]. Reviews of acidic and thermostable amylase production further note that downstream recovery costs scale with enzyme instability during processing, reinforcing that purification strategy and enzyme engineering are complementary rather than independent design choices [23,3]. Table 2, under this section, compares these purification approaches.

Industrial Application in Starch Liquefaction and Saccharification

In the classical starch-to-syrup process, gelatinised starch is first liquefied by thermostable α-amylase at high temperature to yield low-molecular-weight dextrins, after which saccharifying enzymes—glucoamylases and debranching pullulanases—convert these dextrins into fermentable glucose [25]. Pullulanases specifically cleave the α-1,6 branch points of

amylopectin that α-amylase alone cannot fully resolve, and their inclusion substantially raises final glucose yield in industrial saccharification [25]. Synergy among multiple native enzymes secreted by industrial fungal strains, such as *Aspergillus niger* O1, has been shown to improve overall starch conversion efficiency beyond what any single enzyme achieves [26], while thermostable glucoamylases such as that from *Bispora* sp. MEY-1 offer a broad operating pH window that improves compatibility with upstream liquefaction conditions [15]. The engineered BLA and *Geobacillus* variants described above have been evaluated directly in corn-starch liquefaction and related food-processing contexts, indicating a practical pipeline from laboratory-scale mutagenesis to processing-relevant performance gains [7,22]. Collectively, these findings show that the combination of a thermostable liquefying amylase, a compatible debranching enzyme, and an efficient recombinant production and purification pipeline underlies most modern industrial starch-processing operations [1].

Conclusion

Published work on thermostable amylases from *Bacillus licheniformis*, *Geobacillus stearothermophilus*, *Pyrococcus furiosus*, and related organisms demonstrates that no single factor—source organism, expression host, or engineering strategy—alone determines industrial suitability. Instead, the strongest outcomes reported in the literature combine a naturally thermostable scaffold, a secretion-competent recombinant host such as *Pichia pastoris* or *Bacillus subtilis*, and structure-guided mutagenesis validated against real thermal-challenge and kinetic data. Purification strategies that exploit the enzyme's own thermostability, or self-immobilising fusion technologies, further reduce downstream processing costs. Continued integration of computational design with empirical expression and purification data, as reviewed here, is likely to keep extending the operating range and cost-effectiveness of amylases used in starch liquefaction and saccharification.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Golob M conceived the review, conducted the literature search, and drafted the manuscript. Rantanen M contributed the protein-engineering context and critically revised the manuscript. Conti F contributed the bioprocessing and purification framing and approved the final version for submission.

Ethics Statement

Not applicable. This is a narrative review of previously published enzyme expression and purification literature and did not involve new experimental data, human participants, or animal subjects.

Data Availability

No new data were generated or analyzed for this review. All sources synthesized are cited in the reference list and are available through their original publishers.

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