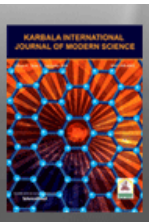

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Systematic review on isolation, purification, characterization, and industrial applications of thermophilic microbial α- amylases

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Abstract

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Keywords

α-Amylase, Food, Paper, Soap, Textile

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REVIEW ARTICLE

Systematic Review on Isolation, Purification, Characterization, and Industrial Applications of Thermophilic Microbial α-amylases

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Abstract

The α-amylase enzyme, sourced from diverse organisms, including plants, animals, and bacteria, plays a crucial role in multiple industries, notably food processing sectors like cakes, fruit juices, and starch syrup. Research identifies thermophilic organisms as prime sources of this enzyme thriving at temperatures ranging from 41 °C to 122 °C. The enzyme purification was carried out using liquid−liquid extraction, which involved the exchange of substances between two liquid phases that were immiscible or partially soluble. The optimal temperature for α-amylase was 45−90 °C. The best pH for bacterial and fungal α-amylases ranged from 5.0 to 10.5 and 5.0 to 9.0. Based on industrial application, de-sizing, scouring, stone washing, bleaching, dying, printing, and polishing were all processes that used α-amylase in the textile industry's finishing phase. The paper business also used α-amylase to modify coated paper starch, while the soap industry applied it to boost the detergency of laundry bleaching compositions and bleach without color darkening. This review underscores the potential of thermostable α-amylase enzymes from thermophilic microorganisms, highlighting their unique high-temperature tolerance properties and broad applicability in food sectors, textiles, paper production, and soap manufacturing.

Keywords: α-amylase, Food, Paper, Soap, Textile

1. Introduction

An extremophile is a species that can survive outside the typical range of at least one environmental element. This signifies that the organism's optimum temperature is not found in its natural habitat [1,2]. Various molecular techniques have been discovered to help extremophile organisms survive in ecological niches characterized by extreme conditions such as high temperatures, pH, salt concentrations, and pressure. This leads to activating metabolic pathways and enzymes, which operate normally at conditions ranging from −5 °C to 130 °C, pH 0−12, 35% salt content, and pressures up to 1000 bar [3,4].

The generation time of microorganisms is significantly short, and they are one of the main sources of

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α-amylase. Based on previous investigations, thermostable α-amylase could be found in the thermophilic, extremophilic, and mesophilic bacteria [5,6], which were optimally achieved at high temperatures. Due to the Generally Recognized as Safe (GRAS) status honored by the U.S. Food and Drug Administration (FDA), *Saccharomyces cerevisiae*, an edible yeast, and other fungi such as *Aspergillus oryzae*, as well as bacteria including *Bacillus licheniformis* and *Stearothermophilus* sp have been used to produce α-amylase, particularly in the food industry [7]. Although there are many types of organisms, those that can survive in temperatures between 41 °C and 122 °C (106 °F and 252 °F) are known as thermophiles [8,9].

Extreme thermophiles, also called "hyperthermophiles," are microorganisms that live at temperatures greater than 80 °C [1,10]. Thermophiles can be categorized in several ways; however, the most useful ones are moderate (50–64 °C), extreme thermophiles (65–79 °C), and hyperthermophiles (80 °C). The thermostable α-amylase are heat resistant due to their unique tertiary structure, thus surviving in these extreme environments [3,4].

Thermophilic microbes produce thermostable enzymes such as α-amylase, which catalyzes the hydrolysis of α-1,4-glycosidic bonds in starch and related polysaccharides at high temperatures. Thermophilic Lipase catalyzes the hydrolysis of fats into fatty acids and glycerol at high temperatures. Thermophilic Protease catalyzes the breakdown of peptide bonds in proteins at high temperatures. Thermophilic Cellulase catalyzes the breakdown of β-1,4-glycosidic bonds in cellulose [10]. Thermostable α-amylase enzymes have wide-ranging applications across various industries, including food, textile, paper, and soap industries [13].

2. Research methods

Online databases such as Science Direct, MDPI, Google Scholar, Springer Link, and Research Gate were used to conduct a literature search for this review. The search approach was centered on principal keywords utilized in different combinations, such as geothermal bacterial, thermophilic bacterial, α-amylase thermostable, geothermal Indonesia, and geothermal Sulawesi. The full-length publications on this topic were published between 2017 and 2023 in peer-reviewed journals.

3. Thermophilic microbes and their α-amylase enzymes

α-amylase, also known as glucan-1,4-α-glucanohydrolase (E.C 3.2.1.1), is an enzyme responsible for starch degradation. It is classified as a calcium metalloenzyme, requiring calcium ions to properly function. Starch molecules are broken down by α-amylase, which helps break down the α-1,4 glycosidic bonds in starch polysaccharides. This enzymatic reaction results in the production of smaller carbohydrate molecules, such as maltose and glucose, which are more readily useable by organisms for energy production and other metabolic processes [11]. α-amylase is obtained from various species, including plants, animals, and bacteria, through industrial extraction and purification [12]. Microbial α-amylase can also be isolated using substrate specificity, repeated dilution, extreme pH, and temperature. Meanwhile, this enzyme is designed and improved through media optimization and genetic engineering approaches for industrial applications [13].

Microorganisms are used as enzyme sources because of their physiologically and physicochemical regulated access, greater product yield, simple recovery in downstream processes, and cost savings in processing [14]. Using microorganisms to express α-amylase is advantageous due to its affordability, flexibility, stability, seasonal insensitivity, and catalytic variability. Microorganisms expressing alpha-amylase are distinguished by their thermostability and halotolerance. In addition, alpha-amylase-producing bacteria can be modified using genetic engineering techniques to produce a more stable enzyme.

It was reported that using bacteria such as *Escherichia coli* as an expression host for yeast proteins can form inclusion bodies (IBs) carrying infectious prions, making fungi the preferred source over other microbial organisms. Post-translational modifications (PTMs) in yeast, a eukaryotic expression host, are more akin to those in higher-level eukaryotes than bacteria [14,15].

Thermophilic bacteria produce an active and stable α-amylase at high temperatures. Starch hydrolyzing enzymes can be produced by different types of *Bacillus*, including *Geobacillus stearothermophilus* [16], *Bacillus subtilis* [17,18], *Bacillus licheniformis* [1], *Anoxybacillus* [7], *Bacillus amyloliquefaciens* [2,19], *Bacillus cereus* [20], and *Bacillus alvei* [6]. Only a few species of *Penicillium*, namely *Penicillium brunneum*, are known to produce fungal α-amylase [21], while others come from terrestrial isolates of the genus *Aspergillus*. A strain of *Aspergillus* spp. yeast is the starting material for the commercial manufacture of α-amylase. *A. oryzae*, *Aspergillus niger*, and *Aspergillus awamori* are the three most popular species used in industrial production [15]. Table 1 presents various types of microbe sources of thermostable α-amylase.

Table 1. Several types of microbe sources of α-amylase thermostable and their optimum temperature.

Enzyme Source	Optimum Temperature (°C)	Types of Microbe	Sample Source	References
Bacillus licheniformis	60	Bacteria	Tuwa hot-spring	[1]
Bacillus licheniformis	60	Bacteria	Geothermal Spring of Odisha	[5]
Bacillus sp.	55	Bacteria	Agro-industrial waste dumping areas	[7]
Bacillus sp.	90	Bacteria	Bora hot Spring	[9]
Bacillus licheniformis	70	Bacteria	Thermal spring mud in Şõrnak	[22]
Bacillus megaterium	90	Bacteria	Pediatric intensive care unit	[23]
Geobacillus sp.	65	Bacteria	Balçova geothermal region	[14]
Bacillus amyloliquefaciens	60	Bacteria	Honey	[19]
Bacillus cereus	60	Bacteria	Indian Ocean Equator region	[20]
Chaetomium thermophilum	70	Fungus	Bhurung geothermal spring	[24]
Bacillus licheniformis	70	Bacteria	Thermal spring mud in Şõrnak	[22]
Pichia pastoris	62	Fungus	Chinese Nong-flavor liquor starter	[25]
Anoxybacillus thermarum	80	Bacteria	Remboken hot spring	[26]
Aspergillus flavus	50	Fungus	Sago humus	[27]
Geobacillus	70	Bacteria	Manikaran hot spring	[28]
PL16	90	Bacteria	Pulu hot spring, Central Sulawesi	[29]
Bacillus caldotenax	55	Bacteria	Likupang Marine Hydrothermal	[30]
Bacillus caldotenax	55	Bacteria	Likupang Marine Hydrothermal	[30]
BHSS10	55	Bacteria	Waepella Hot Spring, Sinjai South Sulawesi	[31]
Geobacillus sp. DS3	60	Bacteria	Sikidang Crater, Central Java	[32]
Bacillus sp.	50	Bacteria	Bora Hot Spring, Central Sulawesi	[33]
Panninobacter phragmatetus	60	Bacteria	Natar Hot Spring, Lampung	[34]

4. Purification of the thermostable α-amylase enzyme

Mass-produced industrial enzymes do not usually require much work after they have been harvested; therefore, manufacturing and preparation are basic. Generally, α-amylase does not need to be purified commercially, but high-purity α-amylase is required for enzyme uses in the pharmacological and clinical sectors. Pure enzymes are also required to investigate structure-function correlations and biochemical properties. Several methods of purifying enzymes have been explored, each with a different set of features unique to the isolated biomolecule [35]. Some ways α-amylase can be purified in the laboratory are ion exchange, reversed phase, hydrophobicity interaction, and gel filtration chromatography. α-amylase extraction methods, on the other hand, often use organic solvents like ethanol, ammonium sulfate, acetone precipitation, and ultrafiltration [36]. Traditional multi-step processes are difficult to repeat due to the high equipment cost used at each stage and are potentially wasteful because of the time and effort involved [37]. Since various aspects of pre-processing procedures can be merged into a single operation, liquid–liquid extraction represents an appealing purifying alternative. A previous report showed that when two immiscible or partially soluble liquid phases were brought into contact with one another, a process known as liquid–liquid extraction occurred. This technique's simplicity, low cost, and scalability have made it popular in the chemical industry [37]. For almost a decade, liquid–liquid extraction has successfully been used to purify biomolecules on an industrial scale. This method produces less viscosity, inexpensive chemicals, and a short-phase separation process. Exploring and understanding the dynamic behavior of these systems is also important to optimize plant-wide control of continuous liquid extraction and evaluate safety and environmental concerns early in the design process [24]. Fig. 1 illustrates the flow chart of the purification process of the α-amylase enzyme.

5. Characterization of the thermostable α-amylase enzyme

It is possible to learn about α-amylase by looking at its temperature and pH stability, as well as its metal ions and chelating reagents, substrate specificity, kinetic constants, inhibitors, and activators. Therefore, when searching for the best microorganisms to use in specific industrial production processes, it is necessary to determine the optimal temperature, pH, and stability. The DNS method [24] is used in each characterization to measure enzyme activity.

5.1. Optimization of temperature and pH

For optimal operations in industries that use α-amylase, there is a need to determine which temperature and pH conditions produce the best results

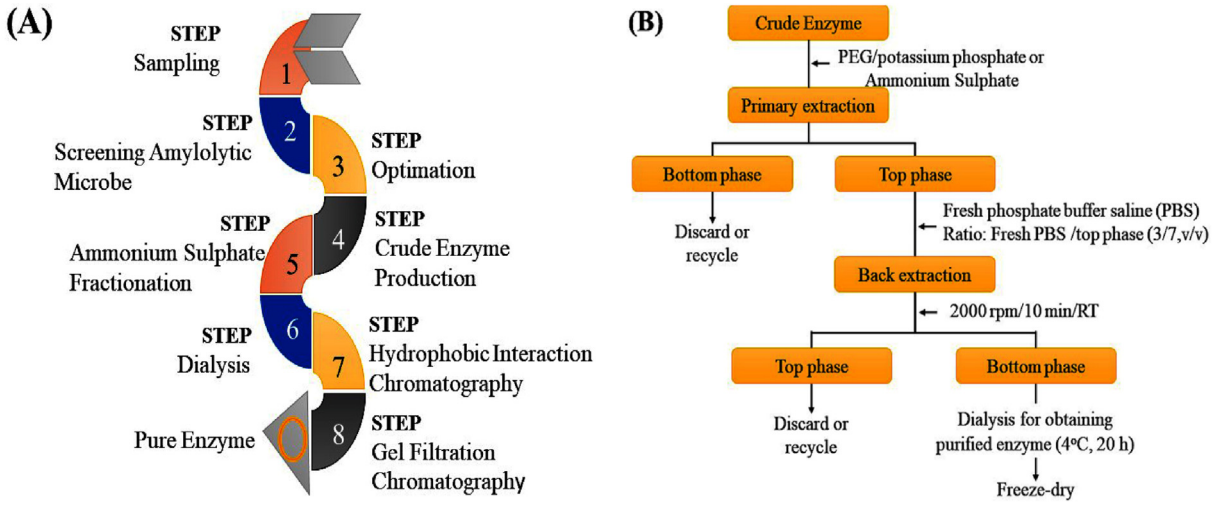

Fig. 1. Flow chart of the standard purification process (A) and liquid–liquid extraction (B) of the α-amylase enzyme.

for the enzymes from *Bacillus licheniformis* B4-423 [41]. Therefore, the optimal temperature for B3 [22] activity is 100 °C, higher than *Streptomyces fragilis* DA7-7 [44] in terms of bacterial α-amylase. The optimal temperature for α-amylase produced by *Komagataella phaffii* is 90 °C, while *Aspergillus flavus* NSH9 and *Trichoderma* are at 50 °C. The optimal pH for bacterial amylases ranges from 5.0 to 10 [22]. Table 2 displays the optimum temperature and pH of α-amylase sourced from various thermophilic bacteria.

The expression hosts of the enzymes, bacteria, and fungi may have different features, leading to optimal temperature variations [42]. Since thermophilic bacteria are better and can withstand high temperatures, the optimal temperature of its α-amylase is also greater [25].

5.2. Thermal and pH stability

The pH stability and thermostability of enzymes are critical because most industrial operations are carried out at temperatures above room temperature and a non-neutral pH. The optimum temperature for α-amylase has been used in most thermostability studies. The results showed that *Anoxybacillus* sp. α-amylase activity peaked at 80 °C during characterization [26]; hence, 70°C-90 °C was selected as the temperature range. The α-amylase expressed by the strain also maintained >49% of its activity after 30 min of incubation at 80 °C, making it a good candidate for use in the starch saccharification process [26,43].

The enzyme retains more than 80% of its activity for pH stability after 210 min of incubation at pH 8.0 and 9.0. Furthermore, after 210 min of pre-incubation at pH 10.0, α-amylase from the strain retains 45% of its initial activity [44]. It was also found that α-amylase produced by *A. flavus* was stable at 50 °C, with 87% of its activity still there after 60 min of incubation [27]. After 24 h of incubation at pH 6.0 and 7.0, the α-amylase enzyme from strain NSH9 retained nearly all of its initial activity [27,45].

The stability of enzymes when held at 30 °C and chilled at 4 °C is significant to be determined, even though α-amylase's thermostability needs to be characterized. According to a study, the recovery of α-amylase, expressed in *Bacillus* sp. using glycerol as a carrier or stabilizer, was higher at 4 °C (114%) than at 30 °C (103%) [46,47]. However, 4 °C is significantly higher than the sample held at 30 °C (30.7%), when only water acts as the carrier and no glycerol

Table 2. Optimum temperature and pH of the α-amylase enzyme from several thermophilic bacteria resources.

Enzyme Source	Optimum pH and Temperature (°C)	Sample Source	References
Bacillus amyloliquefaciens BH072	pH 7 and 80 °C	Honey	[19]
Bacillus licheniformis B4-423	pH 6 and 100 °C	Tangshan and Laoshan hot spring	[38]
Bacillus licheniformis So-B3	pH 8 and 90 °C	Thermal spring mud	[22]
Aeribacillus pallidus BTPS-2	pH 7 and 70 °C	Geothermal spring of Nepal	[24]
Bacillus cereus	pH 8 and 50 °C	Chilika Lake	[39]
Bacillus cereus	pH 10 and 80 °C	Indian Ocean Equator region	[20]
Geobacillus icigianus BITSNS038	pH 6 and 7 °C	Hotspring Surajkund	[40]

stabilizes. These results demonstrate the significance and necessity of shipping α-amylase with glycerol as a stabilizer at a temperature of about 4 °C due to variations in ambient temperatures in different countries [48]. Table 3 presents the thermostability and pH stability of several α-amylase enzymes.

5.3. Activators and inhibitors

α-amylase is an enzyme that breaks down starch. Some metal ions at the right concentration can improve it, while other chemicals and inhibitors can work less well [28]. Since α-amylase is a calcium metalloenzyme, the activity increases when a calcium ion (Ca^{2+}) or salt ($CaCl_2$) is introduced to the reaction mixture. Meanwhile, the activity increases by 8 ± 5% when 4 mM of Ca^{2+} and Hg^{2+} is added to the mixture containing α-amylase generated from *Bacillus licheniformis* [22,28].

Adding 5 mM of Ca^{2+} and Hg^{2+} to a mixture with purified α-amylase from the *T. fontinaldi* strain increased its activity by 55 ± 3.9% [12]. On the other hand, the mercury ion (Hg^{2+}) stops 15 ± 3% of α-amylase's ability to break down amylo groups. This occurs because the enzyme binds to it and sticks together undefinedly [12,49]. Fig. 2 depicts the structural illustrations of α-amylase without and with a calcium ion (Ca^{2+}) activator and zinc (Zn^{2+}) inhibitor.

6. α-amylase enzyme in the industry field

An enzyme is preferred to thermophiles because it is highly stable and attractive for several industrial processes.

It also has greater thermostability and stability under extreme conditions, such as high pH or low water concentrations, which is useful in many applications. Table 4 presents various types of microbe sources of α-amylase for industrial applications.

6.1. Food industry

α-amylase is commonly used in the food industry to make cakes, fruit juices, and syrup made from starch [63]. The starch in flour can be broken down into simpler sugars called dextrins with the help of enzymes and become more amenable to fermentation by yeast. A previous study stated that increasing the fermentation rate and decreasing the dough's viscosity by adding α-amylase might increase the volume and texture of the final product [28,53]. The additional sugars produced during fermentation enhance the bread's flavor, crust color, and baking quality. α-amylase also prevents the bread from stalling, helps baked goods retain a soft texture, and lengthens its shelf life. *Bacillus stearothermophilus* currently produces a thermostable maltogenic amylase that is widely used in commercial baking [54,64]. Fig. 3a illustrates the application of enzymes in the food industry.

In order to manufacture chocolate syrup, amylase is combined with cocoa pulp, which dextrinizes the chocolate starch and prevents the syrup from thickening [65]. Amylolytic enzymes can also make a cocoa-flavoured syrup with a high cocoa content, excellent stability, and flowability at room temperature, with pH ranges from 5.5 to 7.5 [66]. The syrup is prepared by combining cocoa powder, sugar, and

Table 3. Thermal and pH stability of the α-amylase enzyme from several thermophilic bacteria resources.

Enzyme Source	pH and Temperature optimum	Sample Source	References
Bacillus amyloliquefaciens BH072	7 and 60 °C	Honey	[19]
Bacillus licheniformis B4-423	6 and 90 °C	Tangshan and Laoshan hot spring	[38]
Bacillus licheniformis So-B3	pH 5 and 70 °C	Thermal spring mud	[22]
Aeribacillus pallidus BTPS-2	pH 7 and 80 °C	Geothermal spring of Nepal	[24]
Bacillus cereus	pH 8 and 73 °C	Chilika Lake	[39]
Bacillus cereus	pH 10 and 80 °C	Indian Ocean Equator region	[20]
Geobacillus icigianus BITSNS038	pH 6 and 70 °C	Hotspring Surajkund	[40]

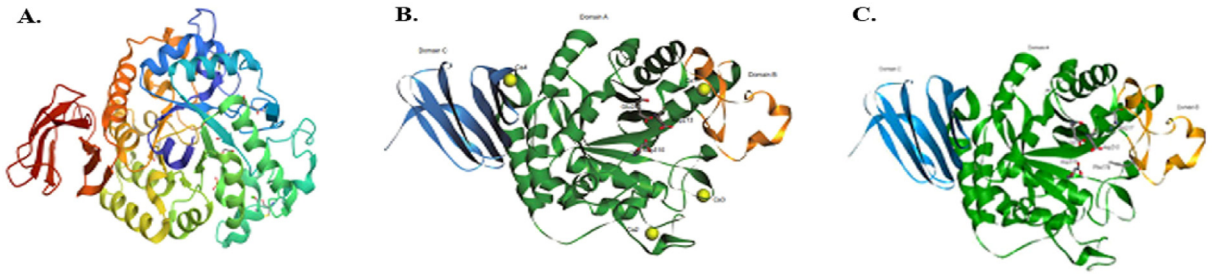

Fig. 2. Structure of α-amylase (A) [50], α-amylase with Ca^{2+} (B) [51], α-amylase with Zn^{2+} (C) [52].

Table 4. Several types of microbe sources of α-amylase for industrial application.

Source	Temperature (°C)	Industrial Application	Reference
Tepidimonas fonticaldi	80	Detergent	[49]
Thermotoga petrophila	98	Noddle	[53]
Bacillus licheniformis	70, 87	Bakery	[28]
Geobacillus bacterium	80	Bakery	[54]
Thermotoga petrophila	100	Textile	[55]
Bacillus subtilis	55	Textile	[56]
Bacillus licheniformis	70	Textile	[57]
Bacillus sp.	60	Paper	[58]
Bacillus cereus	60	Paper	[59]
Actinomadura keratinilytica sp.	70	Detergent	[60]
Exiguobacterium sp.	45	Detergent	[61]
Geobacillus sp.	50	Detergent	[62]

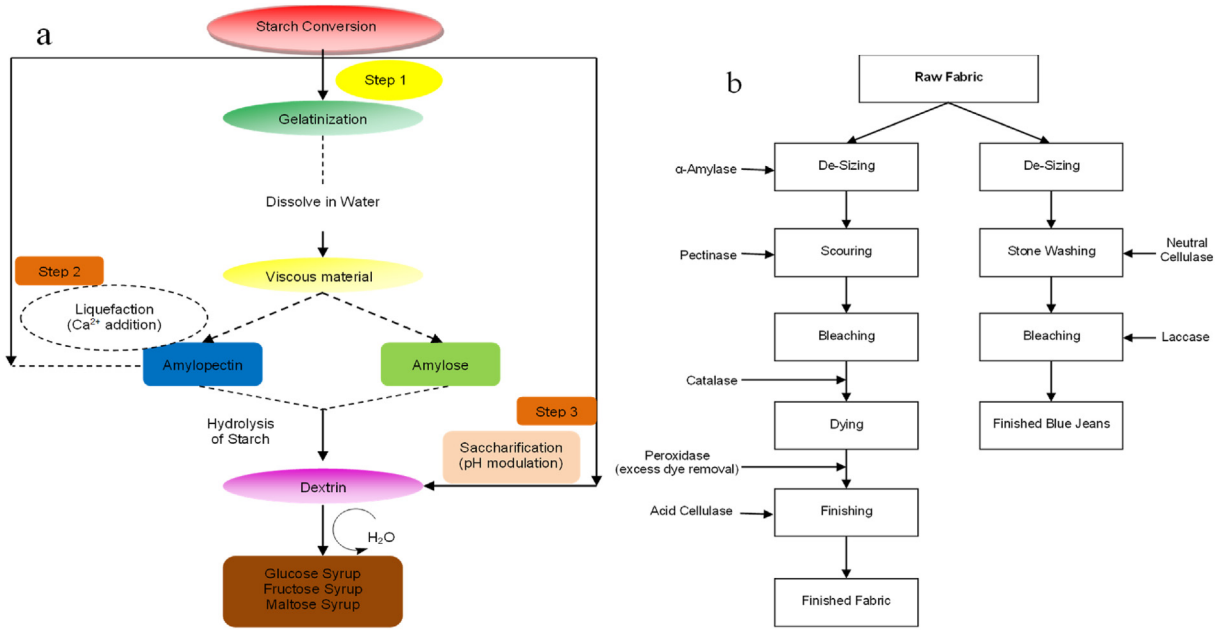

Fig. 3. Application of enzymes in the food industry (a), schematic outline of textile processing and application of enzymes in the textile industry (b).

enough water to reach the desired solids content of 58–65% by weight and stirring in amylolytic enzymes. This is followed by heating to 175–185 °F for at least 10–15 min, increasing the temperature to 200 °F, and allowing it to cool [67]. Meanwhile, a stabilized cocoa-flavored syrup must be added to regular non-acidic candy mixes at room temperature to manufacture chocolate-flavored candies that harden slowly in the freezer [63,65].

6.2. Textile industry

An enzyme is an alternative biocatalyst for textile manufacturing that converts synthetic materials into clothing [55]. Desizing, scouring, stone washing, bleaching, dying, printing, and polishing are all procedures included in the finishing process [73,74]. Meanwhile, fabrics' texture, quality, strength, and market value can be significantly improved using enzymes such as cellulase, amylase, catalase, pectinase, laccase, etc. [55]. Desizing with amylase removes starch; bio-scouring with pectinase can eliminate non-cellulosic pollutants; cellulase makes fabrics softer and more water-repellent; and catalase and laccase make fabrics more retentive. It was also reported that peroxidase and catalase could break down synthetic colors and eliminate excess oxidants during bleaching [56].

An enzyme is distinguished by a complex organic structure, effective catalysis, solubility, specificity, and lack of environmental toxicity because of the biological catalyst's status [56]. Generally, the textile industry frequently uses enzymatic de-sizing processes [68]. The enzymes used include amylase, maltase, dextranase, and cellulose from diverse biological sources to break down and remove

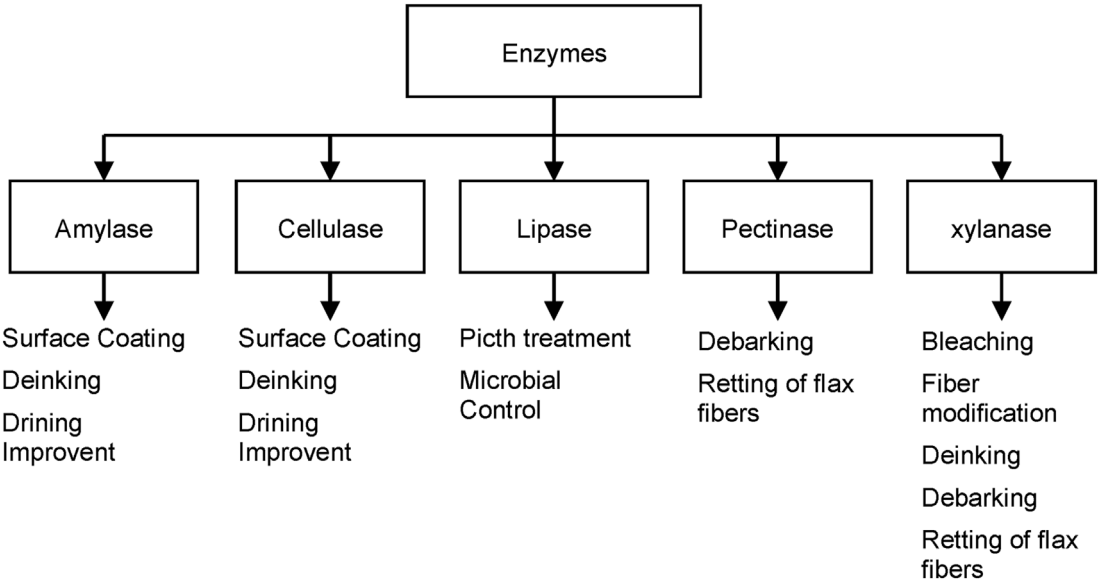

Fig. 4. Application of various enzymes including amylase used in the paper and pulp industries and their specific functions.

components, increasing the size of the material, mainly starch, from textiles [68]. Fig. 3b illustrates the schematic outline of textile processing and the application of enzymes in the textile industry. Unlike the acid and oxidative approaches, enzymatic de-sizing does not damage the cloth while destroying the sizing substance. This is because the starch in polystyrene, silk, and cotton materials is efficiently sized or removed with the help of amylase. Therefore, the entire de-sizing process is designed for the specific enzyme concentration and temperature [69].

The maximum fabric size is achieved through starch removal, which necessitates optimizing enzyme concentration, treatment duration, and fabric temperature [69,70]. The weight of starch-coated fabrics is compared before and after they are subjected to enzymatic treatment in settings that are optimum for starch removal to analyze the created fabrics. Therefore, the starch-coated fabrics are first incubated separately for 0.5–8 h at intervals of 0.5 h. Starch elimination from polystyrene, silk, and cotton fabrics all reaches 70%, 75%, and 85%, respectively, while using the enzymatic approach [57].

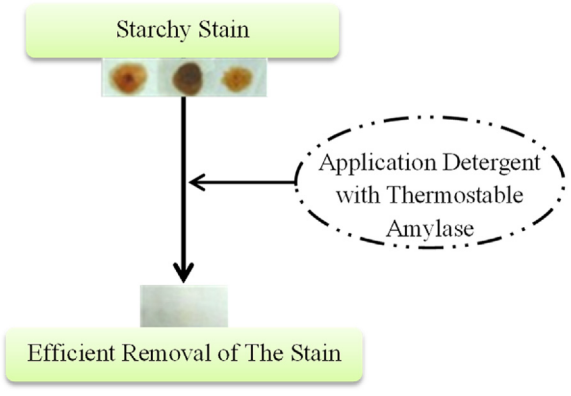

Fig. 5. Diagram schematic of application of α-amylase enzyme in the detergent industry.

6.3. Paper industry

In the pulp and paper business, α-amylase produces a modified starch for coated paper with high molecular weight and low viscosity [58,71]. This strengthens the paper's surface and makes it easier to write on. Since natural starch has extremely high viscosity for paper size, the polymer can be partially degraded with α-amylase in a batch or continuous process [59]. Starch is a suitable covering for paper and is also used as a sizing agent to improve the paper's quality and era's ability. Due to its size, paper becomes more rigid and durable [59]. Fig. 4 depicts the application of various enzymes utilized in the paper and pulp industries and their specific functions.

6.4. Detergent industry

α-amylase is a crucial enzyme in the production of detergents and is commonly used in laundry bleaching formulas since it boosts detergency and prevents color loss [49,60]. The addition of enzymes to laundry detergent bar formulas helps stabilize the bleaching component and keep it working as intended. Meanwhile, gypsum boards generally used in drywall installation are made from modified starch [60]. Fig. 5 illustrates the schematic diagram

of applying the α-amylase in the detergent industry. The addition of enzymes to the detergent improves the stain-removing power and makes it ecologically friendly [60]. About 90% of liquid detergents contain the second enzyme, namely amylase [61,72]. Meanwhile, cleaning instruments and endoscopes with detergents is a common practice in hospitals [75]. Dishwashers and washing machines use this enzyme to break down the starch left behind by foods such as potatoes, gravy, chocolate, custard, etc., into simpler sugars, namely dextrins and oligosaccharides [62].

7. Conclusions

Thermostable enzymes derived from thermophilic microorganisms are highly favored due to their rapid growth and efficient production capabilities. These enzymes have garnered significant attention for their applications in industrial processes that operate at elevated temperatures. Businesses across various sectors seek out thermostable enzymes because of their ability to maintain activity under extreme heat conditions. Thus, the α-amylase enzyme, highlighted in this review, is extensively utilized in the food, textile, paper, and soap industries due to its remarkable heat resistance and functional versatility. Exploring novel thermophilic microorganisms in diverse environments could uncover new α-amylase variants with unique characteristics, further expanding their industrial utility. Integrating these enzymes into sustainable processes, such as biofuel production or bioremediation, represents another exciting avenue for future development. Overall, the future of thermostable α-amylase appears promising, driven by continuous innovation and expanding industrial applications.

Ethics information

This research is not related to ethical issues as it does not involve living organisms.

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