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REVIEW

Challenges and prospects of microbial α-amylases for industrial application: a review

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Abstract

α-Amylases are essential biocatalysts representing a billion-dollar market with significant long-term global demand. They have varied applications ranging from detergent, textile, and food sectors such as bakery to, more recently, biofuel industries. Microbial α-amylases have distinct advantages over their plant and animal counterparts owing to generally good activities and better stability at temperature and pH extremes. With the scope of applications expanding, the need for new and improved α-amylases is ever-growing. However, scaling up microbial α-amylase technology from the laboratory to industry for practical applications is impeded by several issues, ranging from mass transfer limitations, low enzyme yields, and energy-intensive product recovery that adds to high production costs. This review highlights the major challenges and prospects for the production of microbial α-amylases, considering the various avenues of industrial bioprocessing such as culture-independent approaches, nutrient optimization, bioreactor operations with design improvements, and product down-streaming approaches towards developing efficient α-amylases with high activity and recyclability. Since the sequence and structure of the enzyme play a crucial role in modulating its functional properties, we have also tried to analyze the structural composition of microbial α-amylase as a guide to its thermodynamic properties to identify the areas that can be targeted for enhancing the catalytic activity and thermostability of the enzyme through varied immobilization or selective enzyme engineering approaches. Also, the utilization of inexpensive and renewable substrates for enzyme production to isolate α-amylases with non-conventional applications has been briefly discussed.

Keywords α-Amylase · Scale-up · Bioreactor · Immobilization · Protein engineering

Introduction

α-Amylases are the oldest carbohydrases that can replace chemical starch liquefaction in various sectors by the selective breaking of α-1,4 glycosidic bonds (Abraham et al. 2013; Aleem et al. 2018; Egbune et al. 2022). This class of amylases represents almost ~ 25% of the global market share of total enzymes with deployment into the textile, detergent, beverage, paper, pharma, bakery, and biofuel industries (Abd-Elhalem et al. 2015; Kaur et al. 2021; Hallol et al. 2022). The global market size was valued at USD 278.2 million in 2018 and is expected to increase to USD 353 million by 2026 (Niego et al. 2023). α-Amylases for bakery applications will witness the fastest geographical volume gains in the Asia Pacific at a CAGR of 5.9% (https://www.grandviewresearch.com), underpinned by end-user demand to improve the texture of the dough and the color, aroma, and softness of the final bakery products. Also, rising demand from the animal feed industry and discerning consumer needs for products with nutritional value, efficiency, and environmental impact considerations in the pulp and paper industry have helped ramp up global requirements of the enzyme with Novozymes (Denmark), Royal DSM N.V. (Netherlands), AB Enzymes (Germany), and DuPont (USA) being among the major market players (https://menafn.com; https://gminsights.com) (Ahuja and Malkani 2019).

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In the realm of biological taxonomy, a multitude of organisms spanning the plant kingdom (including soybeans, sweet potatoes, barley, wheat, and maize) as well as the animal kingdom (encompassing *Homo sapiens*, canines, and avian species) exhibit the capacity to synthesize α-amylases (El-Gendi et al. (2022). However, when it comes to the production of enzymes, microorganisms are widely regarded as the most exemplary cellular factories due to their remarkable catalytic activity, stability, simplicity of production, and ability to optimize various parameters, as elucidated by Dakhmouche Djekrif et al. (2021).

α-Amylase, a canonical metalloenzyme, crucially relies on the presence of calcium ions (Ca^{2+}) to attain its operational efficacy, stability, and overall structural integrity, as outlined by Nandi et al. (2022). This enzyme, through its interaction with starch, undertakes the conversion of the said polysaccharide into glucose, maltose, and dextrins, as discerned from the findings of Arnau et al. (2020). Notably, α-amylase (EC 3.2.1.1) functions as an endo-amylase, while β-amylase (EC 3.2.1.2) acts as an exo-amylase, and γ-amylase (EC 3.2.1.3) exists as another noteworthy class of amylases. These enzymes exhibit distinct mechanisms of action, executing diverse approaches in their cleavage of starch molecules. Particularly, α-amylase disrupts α-glycosidic linkages at random locations within the starch substrate, a characteristic distinguishing it from its counterparts resulting in maltose, dextrins, and oligosaccharides (depicted in Fig. 1). Conversely, β-amylase selectively targets the maltose-producing α-1,4 glycosidic linkage at the nonreducing end, thereby yielding maltose (Pan 2021). This enzyme is predominantly prevalent in plants and microorganisms (Lahiri et al. 2021). On the other hand, γ-amylase, known for its ability to break both α-1,4 and α-1,6 glycosidic linkages from the non-reducing end, exhibits a broader spectrum of cleavage activity directed towards glucose as the major product (Tong et al. 2021). Of the three classes above of amylases, α-amylases are favored in industrial applications due to their superior kinetic properties. However, their relatively lower selectivity than β and γ-amylases represents a potential trade-off.

This review provides an in-depth detail of the structural attributes of α-amylase, shedding light on various thermodynamic parameters that govern its functional properties and functional stability. It also provides a comprehensive understanding of the challenges associated with scale-up for different industrial applications of the enzyme with possible strategies to combat/mitigate the issues by considering the various factors governing the bioprocess, protein engineering, or enzyme immobilization, for which hardly any literature is available. To the best of our knowledge, this is the first review encompassing almost all the critical factors associated with α-amylase production for industrial application. The scope of α-amylase for possible application as a bioremediating agent as a non-conventional application has also been discussed.

α-Amylase: an insight into its structure

Genome sequencing projects have elucidated the sequence of several microbial amylases, enabling scientists to solve and model their 3D structures (Fig. 1). Conformationally, α-amylase generally has three domains. The catalytic or central domain, A, is the largest and consists of an eight-stranded α/β barrel referred to as the TIM barrel, first discovered in chicken muscle triphosphate isomerase—hence the name (MacGregor 1988). This region carries three active site residues (two Asp and one Glu residue) that are crucial in enabling substrate binding (Fig. 1) (Brayer et al. 1995). One Aspartate residue helps to maintain the integrity of the active site by interacting with the neighboring conserved Arginine through H-bonds. The other Aspartate molecule interacts directly with the substrate (such as starch), resulting in substrate distortion and elevating the pKa of the Glutamate, which acts as the proton donor to the glycosylated oxygen moiety and thereby enables hydrolysis through the formation of a beta-linked glycosyl-enzyme intermediate (Mehta and Satyanarayana 2016). Domain A is present in all α-amylases, whether obtained from bacteria, fungi, or animals.

On the other hand, domain B (a protrusion from domain A) has non-uniform β-sheets compared to the TIM barrel of domain A. It constitutes a significant portion of the substrate binding pocket of various α-amylases and differs significantly in conformation (Li et al. 2017a, b). It appears as an insertion between the C and A domains and remains attached to the latter through a disulfide linkage. Interestingly, the B domain is absent in liquefying α-amylases of *B. subtilis*, whereas the region remains slightly elongated and has distinct features in most thermostable bacterial amylases. It is assumed that the rigidity of the B domain enables substantial improvement in thermostability and plays a crucial part in imparting substrate specificity (Zeng et al. 2020). Domain C, the most variable domain, accounts for the C- terminal sequence assembled into a globular unit forming a Greek key motif. It remains loosely bound to the TIM barrel compared to the B domain and at its opposite end. The C terminal domain is structured with β sheets and connects to domain A by simple polypeptide linkage. This region shows significant irregularities in sequence and length between different α-amylases. Though its function is not entirely understood, some researchers have shown that this variable region can play an essential role in enzymatic activity and stability. Fort et al. (2021) indicated that domain C in *Bacillus stearothermophilus* plays a vital role in its enzymatic activity.

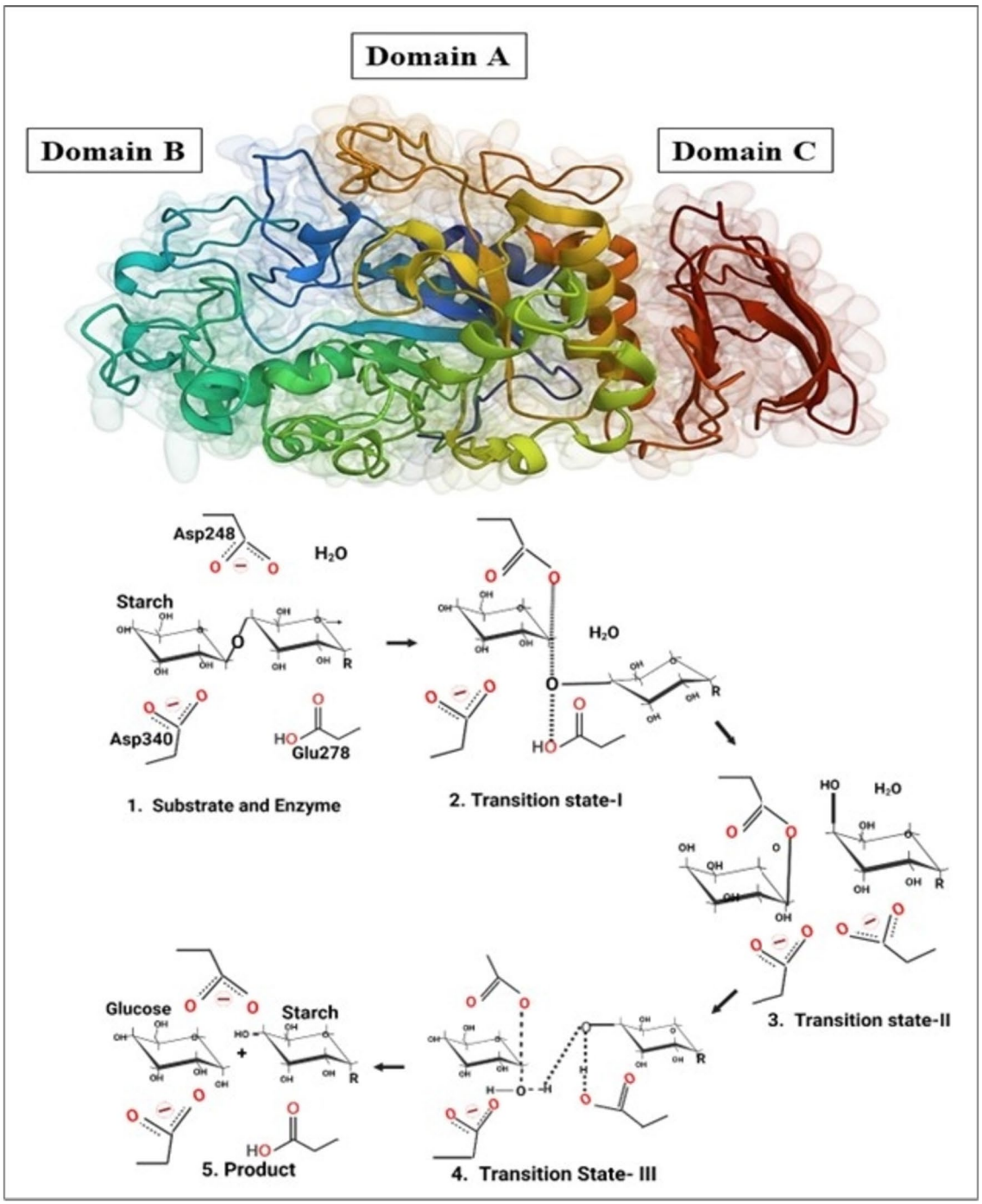

Fig. 1 3-D structure of α-amylase with different domains and mechanistic action of α-amylase from *Bacillus* sp. The single letters indicate standard amino acid codes

Using C-terminal domain truncated mutants (CTDM) of *Geobacillus thermoleovorans* (Zeng et al. 2020), the researchers noted that although the wild type α-amylase could successfully bind the corn starch, the CTDM amylase could not attach to the substrate under identical conditions. Also, its k_{cat} and half-life were reduced by 22.1% and by 1 h, respectively, which indicated that the CTD probably played a part in starch binding and enabled it to prolong the thermostability. The conserved calcium-binding groove is localized in central domain A towards the C terminal region. Mehta and Satyanarayana (2014) confirmed the function of domain C in starch binding by

carrying out truncation analysis; α-amylase produced by *G. thermoleovorans* mutant could not bind the raw starch.

α-Amylases from *Aspergillus awamori* showed the presence of an additional domain (CBM), i.e., a carbohydrate-binding module. Also referred to as an ancillary module comprising 40–200 amino acid residues that facilitate the binding of various polysaccharides (Singh et al. 2021), an α-amylase starch binding domain (SBD) is such a CBM with specificity toward starch (Baroroh et al. 2019). SBD contains a β-sandwich fold and is categorized under family CBM20. A linker region facilitates the binding of CBM with the catalytic domain. It enhances the hydrolysis rate by increasing the concentration of raw starch at the site by adsorption (Cripwell et al. 2020). The amino acid residues W543, W590, W616, and W662 play crucial roles in the binding starch granule. The CAZY database contains ~ 2704 SBD entries (out of 292,679 hits) in amylases produced by *Bacillus cereus, Bacillus circulans,* and *Aspergillus niger* (http://www.cazy.org/). α-Amylase from *Saccharomycopsis fibuligera R64* showed lower adsorption of raw starch due to a lack of a carbohydrate-binding domain (CBD) (Baroroh et al. 2019). α-Amylase from *Bacillus aryabhattai* showed the presence of CBM similar to amylase from soybean and other plants (Duan et al. 2021). SBD is present in various amylases, having the capability to degrade raw starch. These are additional domains apart from the specific domains found in different α-amylases (Mehta and Satyanarayana 2014).

Calcium: an inherent cofactor

Calcium plays a significant role in α-amylase activity. The number of bound metals typically varies from one to ten within the protein molecule (Navjot et al. 2022). On the other hand, Gopinath et al. (2017) reported seventeen binding sites for calcium in *Bacillus amyloliquefaciens*. Although the functions of α-amylases from various origins have changed due to evolution, catalytic residues, and calcium binding pockets are still conserved (Posoongnoen et al. 2021; Marengo et al. 2022). Calcium ions have proven to function critically in α-amylases, resulting in structure, activity, and stability improvement, especially in thermophilic ones (Yadav 2012). It has been noted that the potential residues binding to Calcium are conserved. The metal ions are necessary to maintain protein shape in their functional conformations (by stabilizing the interface of domains A and B) and resist enzymes' thermal inactivation (Liao et al. 2019). The authors reported that with loss of Calcium ions in thermophilic *Anoxybacillus* sp. GXS-BL, the denaturation temperature of the enzyme was substantially reduced by 10 °C, similar to those reported with *Aspergillus oryzae*. Calcium removal from the protein led to increased susceptibility to proteolytic degradation and loss of structural integrity. As an interesting fact, sequence analysis has revealed the presence of a metal triad at the interface of domains A and B, i.e., calcium–sodium–calcium (Ca–Na–Ca) (Machius et al. 1998; Lee et al. 2022) in certain *Bacillus* amylases, which probably account for their increased thermostabilities. The structured presence of sodium and calcium creates a unique arrangement surrounded by negatively charged residues. Ca and Na in the metal triad are responsible for the stability of thermophilic α-amylases (Yi et al. 2018). However, there are one or two reports that calcium ions also negatively impact the enzyme activity in some species. For example, α-amylase from *A. oryzae* is found to be inactivated in the presence of Calcium ions (Ng et al. 2021). The Calcium-binding site exhibits secondary interactions with Asp, Glu, and Asp catalytic residues, leading to the inactivation of the active site. Despite these exceptions, Calcium is an essential component of the enzyme that significantly enhances the catalytic efficiency of α-amylase through stable interactions with various amino acid residues within the protein molecule (Marengo et al. 2022).

Amino acid composition: a key determinant of stability

The amino acid composition within the α-amylases, apart from the catalytic activity, can also significantly influence the enzyme stability. It has been observed that elevated levels of non-polar amino acids, particularly those with hydrophobic properties, result in a reduction of charged amino acids, specifically Arginine and Glutamate residues. The charged amino acids, however, play a crucial role in ionic interactions, salt bridges, and H bondings within the proteins which promote stability. For example, an increase in aromatic amino acids, especially Tyrosine residues, enhances cation–pi interactions. George et al. (2020) performed an in-silico sequence analysis of several α-amylases and observed that certain charged amino acid residues such as Lysine, Arginine, Glutamate, and their corresponding dipeptides occur at a higher frequency in the thermostable versions of the enzyme compared to their mesophilic counterparts. Statistical data analysis has also shown that amino acid substitutions such as Glycine to Alanine and Lysine to Arginine in thermophilic α-amylases are favored. Whereas a decrease in Methionine residues promotes thermostability within a particular protein. It is assumed that thermodynamically, the distinctive compositions of amino acids are closely linked to specific characteristics, notably the shape and Gibbs free energy change of hydration for native enzymes. It is assumed that amino acid composition disparities can be a pivotal determinant of protein thermostability. However, to ascertain the intricate relationship between structure and

function in α-amylases, further exploration of protein activity and stability needs to be carried out and modeled as a function of amino acid composition (across a larger extent in the database) using the advanced machine learning or AI-based) computational tools at the micro level.

Thermodynamics of α-amylase

The thermodynamic parameters for starch hydrolysis using α-amylases have been derived from the transition state theory by Eyring and Stearn.

where,

$$ln\frac{Kd}{T} = ln\left(\frac{k}{h}\right) + \left(\frac{\Delta S^*}{R}\right) - \left(\frac{\Delta H^*}{R}\right)\left(\frac{1}{T}\right) \quad (1)$$

where h = Planck's constant (6.63×10^{-34}), R = gas constant (8.314 J/K mol), T = absolute temperature, ΔS^* = change in entrophy, ΔH^* = change in enthalpy, k = Boltzmann constant (1.38×10^{-23}).

The change in enthalpy provides valuable information for the effectiveness of the transition state. In contrast, a change in entropy suggests the affinity of the substrate for the enzyme and the stability of the transition state. On the other hand, the change in Gibb's free energy (ΔG) is an indicator of the spontaneous reaction. The protein structure strictly regulates both parameters.

$$\Delta S^* = \frac{\Delta H^* - \Delta G^*}{T} \quad (2)$$

Shukla and Singh (2015) performed deactivation studies varying from 50 to 100 °C to analyze the thermostable amylase from an actinobacteria *Laceyella sacchari* TSI-2. The change in entropy was estimated at around − 126.45 J/mol/K, which indicated that α-amylase binds with high affinity with its substrate, soluble starch. Also, the enzyme had a high energy of deactivation (21.16 kJ/mol), which corroborated its stability at elevated temperatures. Nwagu et al. (2020) discovered a high change entropy (~ -180 J/mol/K) by α-amylase from *Paecilomyces variotii ATHUM 8891* while binding with starch. The enthalpy change was around 34.09 kJ/mol, which highlighted that higher free energies were necessary to inactivate the enzyme. In another report, Samanta et al. (2014) described an α-amylase derived from *Bacillus licheniformis* SKB4, which is highly thermostable with an optimum temperature of 90 °C. The free energy for substrate binding (ΔG E–S) and transition state at 90 °C were found to be 5.53 and − 17.4 kJ/mol, respectively. Yandri et al. (2020) reported a thermostable enzyme from *Bacillus subtilis* ITBCCB148 with an optimum working temperature of 65 °C. Native α-amylase had a half-life ($t_{1/2}$) of 1.89 h with a ΔG value of 107.3 kJ/mol.

Interestingly, immobilizing the α-amylase using chitin enhanced its half-life by ~ 12-fold and improved the ΔG value to 115.51 kJ/mol (Yandri et al. 2021). Table 1 highlights the thermodynamic properties of different α-amylases isolated from various bacterial and fungal species. The mesophilic α-amylase counterparts have significantly lower enthalpy and Gibb's free energy values. Based on the thermodynamic parameters, the enzyme performance can be easily deduced.

Production methods

α-Amylase production has been attempted through different fermentation techniques. However, commercially only two production methods, (a) solid-state fermentation (SSF) and (b) submerged fermentation (SMF), have been successful (Elyasi et al. 2020) (Table 2). The conventional fermentation technique SMF employs a free-flowing liquid to grow the culture in an aerobic or anaerobic environment (Bakri et al. 2020). As a homogeneous medium, the substrate is utilized rapidly, and the α-amylase can be efficiently secreted in the liquid broth. This easily enables the recovery and purification of the enzyme for downstream applications. Besides, the physical parameters such as aeration, pH, and temperature can be smoothly regulated, which dictates the enzyme kinetics (Sharma et al. 2016).

SSF, on the other hand, employs a solid substrate such as bran, pulp, leave, peels, or raw biomass that is biodegradable for the growth of microbe at a solid–liquid interface (Cuadrado-Osorio et al. 2022). Unlike SMF, the substrates are utilized slowly but steadily in this method. The process does not require specialized equipment, resulting in a high concentration of products with minimal effluent generation (Ramachandran et al. 2010; Srivastava et al. 2019). However, yeast and fungi were considered most appropriate for SSF compared to bacterial cultures with their substantial water requirements (Prabhu et al. 2022). Also, bacterial cultures can be well maintained and exploited for SSF processes, as shown by recent research (Tsegaye and Gessesse 2014). A comparison of the two methods has shown that SSF is more appropriate for developing countries because of its cost-effectiveness with relatively little energy expenditure as compared to SMF.

Although the cost of α-amylase production is the most crucial factor for any process development with the enzyme, it is usually not taken into serious consideration. Very little information is available on α-amylase production costs, and the authors mostly provide an estimated price (US $3/kg to US $50/kg) without giving detailed information about the source or the models (Sóti et al. 2018). Among a few handfuls of reports available, Castro et al. (2010) estimated the cost of α-amylase production from *A awamori* using SSF of

Table 1 Kinetics of mesophilic and thermostable α-amylases

Sr. no.	Organism	Feed	Temp (°C)	pH	Half-life ($t_{1/2}$) (min)	V_m (U/mol)	K_m (mg/mL)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/mol/K)	E_d (kJ/mol)	Remarks	References
	Mesophilic α amylase												
1	*B. licheniformis* RT7PE1	Raw starch	55	9.8	1020	19.5 U/mg	3.4	− 164.8	44.8	− *155.6*	45.2	High enzyme stability influenced by Ca^{2+} ions	Tabassum et al. (2014)
2	*A. awamori*	Olive oil cake	50	7.0	289	19.8 U/mg	0.0373	*NR*	NR	*NR*	8.71	T_{Opt} increased to 55 °C	Karam et al. (2017)
3	*Rhizobium* sp strain A1	Lemon peel	40	5.0	264.3	2.3	15.4	90	65.2	92.25	67.87	$t_{1/2}$increased to 346.6 min due to less unfolding	Abdul-Hameed et al. (2022)
4	*A. fumigatus*	Soluble starch	55	4.0	40.53	1.09×10^{-5}	3.04	104.4	–	*NR*	*NR*	1.5-fold stability improvement	Yandri et al. (2023)
5	*A. carbonarius*	Raw potato starch	30	5.0	526.2	3.37×10^{-5}	0.34	92	29.9	− 179.7	32.8	Citraconic anhydride modification Increased $t_{1/2}$ by 7.9-fold	Nwagu et al. (2020)
	Thermophilic α-amylase												
6	*B.licheniformis* SKB4	Starch	90	6.5	10	1.05 µmol/mg/min	6.2	5.53	NR	*NR*	31.53	High thermostability with metal independent activity	Samanta et al. (2014)
7	*L. sacchari* TSI-2	Starch	70	7.0	1080	7.589	2.71	78.54	35.32	− 126.45	*NR*	Residual activity increased to 190.63%	Shukla and Singh (2015)

Table 1 (continued)

Sr. no.	Organism	Feed	Temp (°C)	pH	Half-life ($t_{1/2}$) (min)	V_m (U/mol)	K_m (mg/mL)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/mol/K)	E_d (kJ/mol)	Remarks	References
8	*A. rupiensis*	Starch	80	8.0	*NR*	3124	0.58	*NR*	*NR*	− 171	*NR*	Threefold higher production of amylase	Kikani et al. (2019)
9	*G. thermoleovorans*	Potato starch	80	5.0	2100	2778 µmol/mg/min	0.315	*NR*	*NR*	*NR*	97.4	–	(Mehta and Satyanaraya(na (2015)
10	*B. stearothermophilus*	Starch	70	5.0	8	53,000 U/mg	2.1	*NR*	*NR*	*NR*	*NR*	N193F/S242A increased $t_{1/2}$ by 26-fold	Li et al. (2016)
11	*Thermotoga petrophila*	Starch	65–75			2.1 mg/min	3.6	*74.0*	*39.9*	*− 92.3*	*42.9*	Thermostable with high rapid starch hydrolysis	Hameed and Ul-Haq (2020)
12	*Paecilomyces variotii* ATHUM 8891	Starch	60	5.0		58.5 µmol/mg/min	1.1	*7.9 ± 0.6*	*68.4 ± 1.5*	*− 181.2 ± 1.2*	*10.7 ± 0.8*	Stable at acidic pH range (3.0–5.0)	Apostolidi et al. (2020)

NR not reported

Table 2 α-Amylase production by fermentation methods

Sl no.	Microorganism	Method	Substrate	Activity (U/mL or U/g)	Time (h)	Temp (°C)	Pros	Cons	Remarks	References
1.	*B. licheniformis MTCC1483*	SMF	Soluble starch	21.6	24	30	Rapid production of the enzyme	Downstream processing	Stable at pH 8.0, useful in detergent application	Dahiya and Rathi (2015)
2.	*B. licheniformis MTCC 2617*	SMF	Starch	2.59	72	37	Cheap carbon source	Low yield	Activity declined after 3rd day	Anand et al. (2022)
3.	*Bacillus* sp.	SMF	Deoiled cake of groundnut	5211	24	37	Economic agro waste	Parameter control	Enzyme stable at 70 °C and pH 7.5	Simair et al. (2017)
4.	*B. subtilis* SUNGB2 and *B. licheniformis* HULUB1	SMF	Solid food waste	22.14	48	45	Mixed culturing enabled the production of two different varieties of α-amylases	Grinding/blending of feed is required	The enzyme combination enabled the rapid degradation of food waste	Msarah et al. (2020)
5.	*Bacillus cereus*	SMF	Soluble starch	2.56 U/mL	24	35 °C	Minimum requirement and less time is required	Low yield	Optimization resulted in 1.3 fold increase in amylase activity	Raplong et al. (2014)
6.	*A. oryzae* (MTCC3107)	SSF	Deoiled cake of groundnut	9868.12 U/gds	108	32.5	Agro waste as a low-cost substrate	Optimized only for given strain and substrate	Production increased by 11% after scaling up	Balakrishnan et al. (2021)
7.	*B. amyloliquefaciens* (MTCC1270)	SSF	Wheat bran	14.25 ± 0.24 U/mg	48	37	Cheap substrate	Scale-up	Supplementation of inositol and mannitol increases enzyme production	Saha et al. (2014)
8.	*B. subtilis D19*	SSF	Wheat bran	640 U/g	–	–	Ease of availability	Controlled environment	Statistical optimization for improvement	Almanaa et al. (2020)
9.	*B. subtilis* sp. KR1	SSF	Wheat straw	69.37 ± 0.932 U/gds	48	40	Active within a wide pH (5.0–9.0) and temperature range (30 °C–45 °C)	Pretreatment of substrate is required	Agro-industrial wastes can be easily degraded	Pranay et al. (2019)
10.	*Thermomyces* sp.	SSF	Soy and bread waste	39.9×10^3 U/gds	120	55	500% increase in productivity by following a sequential batch operation	Highly dependent on inoculum volume		Cerda et al. (2016)

The italics are used to represent the scientific names of the various microbial varieties for production of alpha amylases

Babassu cake. The initial cost of production was estimated to be very high. With much optimization and strategic planning, the authors brought down the cost to US$ 10.4 kg of the enzyme (by selling the fermented cake as a co-product). In a more recent study, Balakrishnan et al. (2021) performed the production of α-amylase with scale-up (in a 600 L fermenter) from *A oryzae* using solid-state fermentation of edible cakes. Using statistical design and optimization tools, the production could be enhanced by ~ 12%. The economic analysis demonstrated that the partially purified enzyme had a tentative production cost of ~ US$ 7.59/L of the enzyme with very high activity (9868.12 U/g dry substrate). In an interesting study, reported by authors from Bangladesh, an in-house α-amylase could be produced through SSF at a production cost of US$ 0.54/L the enzyme which was ~ 10 times lower compared to the conventional α-amylase (Khalid-Bin-Ferdaus et al. 2018). Such a low-cost enzyme, if successfully scaled up, would be an instant hit for application in the textile and apparel industry.

α-Amylase: a host of endless opportunities

Starch conversion

The α-amylases are essential enzymes with vital applications in starch liquefaction (Paul 2016). The process of starch degradation involves three steps, namely a) Gelatinization, b) Liquefaction, and c) Saccharification (El-Fallal et al. 2012). During gelatinization, starch feedstock, corn, wheat, or cassava undergoes thorough cleansing and pulverization, yielding a fine powder. This meticulous step augments the surface area of the starch particles, ensuring enhanced accessibility for subsequent enzymatic actions. The solid starch slurry is next treated at high temperatures (> 100 °C) to ensure the removal of lipid-starch complexes. This step results in a dense suspension of starch dissolved in water, which enables the α-amylases to partially hydrolyze the polymer into short-chain dextrin, significantly reducing the solution viscosity. This is followed by the enzyme's breakdown of the oligomeric dextrin into reducing monomeric sugars D-glucose and D-fructose. The incubation period and the enzyme loading predominantly govern starch hydrolysis efficacy. Since the current industrial amylases cannot sustain such high temperatures, the gelatinization and liquefaction steps must be performed separately (Silano et al. 2018). The liquefaction process after gelatinization commences with a heat-intensive step, wherein the starch slurry, accompanied by water, is subjected to elevated temperatures. Simultaneously, thermostable α-amylase enzymes are introduced into the mixture. These enzymes catalyze the hydrolysis of α-1,4 glycosidic bonds, effectively deconstructing the starch molecules into shorter dextrin chains. Operating within the 95–105 °C temperature range, this phase facilitates optimal enzymatic activity. After the liquefaction stage, the temperature of the starch slurry is carefully decreased, accompanied by the introduction of additional enzymes. This crucial step encompasses the synergistic action of glucoamylase and α-amylase enzymes. Their concerted effort progressively disintegrates the dextrin chains into individual glucose molecules. The glucoamylase enzyme contributes to the hydrolysis of both α-1,4 and α-1,6 glycosidic bonds within the dextrins, liberating glucose. Saccharification predominantly transpires within a temperature range of 55–65 °C.

Once the desired glucose concentration is attained, meticulous control over the enzymatic activity is exercised by either elevating the temperature or adjusting the pH of the slurry. This serves the dual purpose of halting further enzymatic degradation of the starch and preserving the glucose for subsequent fermentation endeavors. However, there is a search for an enzyme that can sustain even higher temperatures and low pH so that the gelatinization and liquefaction steps may be performed simultaneously (which is still a great challenge in the industry to date).

Detergent industry

α-Amylases are the most used enzyme after proteases in the liquid detergent industry (Dakhmouche Djekrif et al. 2021). They are supplemented explicitly to the laundry and dishwashing detergents for the liquefaction of starch and breakdown of starchy stains from different food preparations and gravies. It principally breaks down the starch into tiny sugar molecules lifted from the clothes during the detergent wash. Additionally, the enzyme prevents swollen starch from adhering to the surface of laundry and glassware (Gürkök 2019). They are generally used in combination with proteases, which boost cleaning (in different industrial sectors) and restore the whiteness of clothes. The amylases that can function at low temperatures and in an alkaline pH range are significant in the detergent industry (Hamid et al. 2022). The Novozyme-developed Stainzyme Plus Evity 24T® and Amplify Prime 100 L® perform exceptionally well in cold washes and even in the presence of strong chelators (https://biosolutions.novozymes.com). One of the significant issues of α-amylase in the detergent industry is its oxidant sensitivity and calcium dependency (Samanta 2022). Genetic engineering has been tried to overcome the limiting issues with pH/temperature stability and calcium dependency. Yang et al. (2017) mutated α-amylase obtained from ciliated protozoan by introducing proline residues in the surface loop and changing valine to threonine near the catalytic site. It increased half-life ($t_{1/2}$) by twofold at 50 °C compared to the wild type. Gai et al. (2018) created a double mutant α-amylase by deleting 179R and 180G from *Bacillus stearothermophilus*.

Half-life was increased by 1.375 fold, and the mutant was stable at a lower pH than the wild type. Farooq et al. (2021) prepared an oxidant-resistant amylase by replacing methionine with leucine at 197 in (*B. licheniformis* α-amylase) BLA, resulting in better oxidative stability. Mutation of oxidation-prone M residues with I, A, and T resulted in improved catalytic activity and oxidative stability. Oxidative stability was increased by 5.4-fold due to mutant α-amylase (M145I-214A-229 T-247 T-47I) (Yang et al. 2013). Natalase® and Termamyl® are some of the amylases available commercially and widely used in the liquid detergent industry (Mehta and Satyanarayana 2016). *B. licheniformis* derived Termamyl®300L is a thermally stable α-amylase that hydrolyses starch at 95 °C (Guerrero-Navarro et al. 2019). Guerrero-Navarro et al. (2022) described an enzyme formulation having two commercial enzymes, Savinase® and Termamyl® Ultra 300 L, which could efficiently remove (~ 75%) fouling in a spray dryer and pilot-scale plate heat exchanger in a dairy industry, which was comparable to the chemical cleaning methods used conventionally in industries based on 1.2 mL/L protease and 1 ml/L Termamyl®300L. α-Amylase and protease are used to remove dirt from household streams and various industrial waste streams. Nowadays, oxidation resistant α-amylases are essential for application in the detergent industry as native α-amylases are sensitive to oxidation (https://www.creative-enzymes.com).

Bioethanol production

The rapid decline of fossil fuels (due to the increasing population) coupled with the issue of climate change has prompted the scientific fraternity to develop clean and renewable biofuels such as ethanol (Khan et al. 2021; Pham et al. 2022). Starch is the preferred carbon source for producing 1G ethanol due to its global availability. α-Amylases readily break down the starch (after liquefaction) into its constituent monomers, which are then rapidly converted by a fermenting yeast such as *Saccharomyces cerevisiae* (Singh et al. 2022) (Fig. 2). Kumar and Singh (2016) reported efficient ethanol production from corn-based starch using a novel vacuum-assisted fermentation method through SSF. Using commercial α-amylases with high enzyme activity (6400 µmol maltose/min mL) and a superior industrial fermenting yeast termed "ethanol red" (*S. cerevisiae* yeast, Lesaffre Advance Fermentation), 18% v/v ethanol titers were achieved. The vacuum-assisted SSF technique enabled rapid ethanol recovery within the system, allowing the yeast to ferment with high productivity without any toxic effects of the product alcohol at higher concentrations (Kumar and Singh 2016). Krajang et al. (2021) reported ethanol production in a single step by combining starch hydrolysis and fermentation plus scale-up studies (3000 L fermenter) from raw Cassava. Using a commercial α-amylase Stargen TM002®, 80.19% of raw Cassava starch was hydrolyzed by yielding ~ 176.4 g/L fermentable D-glucose. The "baker's yeast" successfully

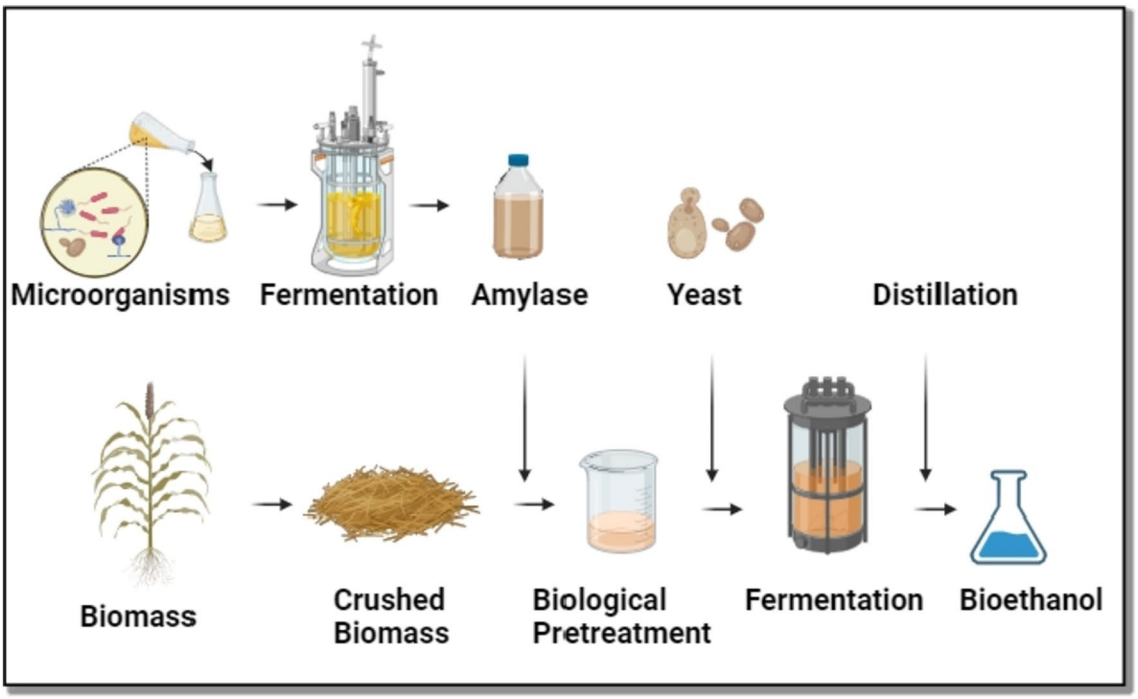

Fig. 2 1G ethanol production process involving α-amylase

fermented the broth with a high yield (8.97% v/v) and productivity (0.98 g/L/h). Since starch hydrolysis requires high-temperature hydrolysis followed by fermentation by mesophilic yeasts, a significant amount of cooling water is needed to lower the temperatures, which consumes energy, thereby increasing process costs. Yeasts with high-temperature ethanol fermenting capability, such as *Kluyveromyces,* would be a promising candidate for such conversions (Bilal et al. 2022). However, their ethanol fermenting capabilities need further improvement to be at par with the industrial *Saccharomyces* sp. Apart from the aforementioned points, bioethanol production faces several challenges: feedstock availability and cost, energy and water requirements, feedstock conversion efficiency, policy, and market factors. Ethanol production also needs to factor in environmental impact issues, such as increased water usage, emissions of greenhouse gases, and potential competition for land with food production. Mitigating these environmental impact elements and ensuring sustainable practices throughout the ethanol production lifecycle is essential for this sector.

Textile industry

α-Amylases are employed in the textile industry for designing and finishing fabric. Starch is applied to yarn before fabric generation as a strengthening agent (to avert splitting of a warp thread during the weaving procedure), typically ensuring a rapid and effective procedure. A wet treatment process later eliminates it from the fabric. The traditional processes of desizing involved using acid or alkali as the desizing material. This damages the cellulose fibers and lowers the cloth's tensile strength (Vaibav et al. 2022). The harsh chemical treatments involve colossal energy expenditure and cause environmental concerns in the textile industry, promoting the utilization of enzymatic desizing. The α-amylases penetrate the space between fibers and selectively remove the starch without adversely affecting the fabric (Al-bedak et al. 2022). The α-amylases from *Bacillus sp.* are nowadays widely used in the textile industries. Enzyme solutions such as Aquazyme 210L® and Termamyl® typically operate between 30 °C-60°C and efficiently remove starch with colossal water and energy savings within a pH range of 5.5–6.5 (https://biosolutions.novozymes.com). Another commercial enzyme, SuperLIQ™®, has been developed by the Bestzyme® Corporation to operate across a broad range of pH and temperatures, functioning on all starch-based sizes (https://www.bestzyme.com/).

Paper industry

α-Amylases are applied in the paper factory to modify the starch-layered paper. Starch is one of the cheapest and most crucial wet-end additives used in the paper industry. The coating or overlaying process makes the exterior of the paper polished, glossy, and robust (Paul 2016; Xu et al. 2018). Also, as a sizing agent, starch adds to the reusability and enhances paper quality. The cohesiveness of natural starch makes it unsuitable for direct application in the paper industry as a sizing agent. α-Amylases are employed for partially degrading starch-coating of paper to improve the viscosity and concentration to achieve the desired consistency (Gangadharan et al. 2020). Specific modifications in α-amylase can increase the viscosity, film-forming properties, and adhesion of the starch-based coating. Deinking is the process of removing ink from recycled paper fibers to produce high-quality recycled paper. Genetically modified amylases can be utilized to enhance the deinking process by breaking down the starch and carbohydrate-based components of ink and improving the efficiency of ink removal. Genetically modified amylases can also be used to modify the surface properties of paper fibers. By targeting the starch components present in the fibers, amylases can alter their structure, making them more receptive to various treatments such as sizing, coating, and dyeing. This modification improves the overall quality and performance of the paper. Bleaching is a crucial step in paper production to achieve the desired brightness and whiteness. Genetically modified amylases can assist in pulp bleaching by degrading starch residues that may interfere with the bleaching process. This ensures better penetration of bleaching agents, leading to improved color removal and brightness. α-Amylase G995® (Enzyme Biosystems, USA), BAN® (Novozymes), and Termamyl® are the few commercial amylases used in the paper and pulp industry (Mehta and Satyanarayana 2016). They also provide stiffness and strength.

Challenges and opportunities

α-Amylase holds great significance in various industrial sectors due to its versatile applications. However, the pursuit of advancements in this field is driven by the challenges posed by specific industrial demands and the opportunities to overcome them. Challenges include ensuring stability under harsh chemical conditions, optimizing substrate specificity, achieving cost-effective production, and addressing limitations in immobilization techniques. On the other hand, opportunities lie in enhancing reactivity and stability through genetic and protein engineering, tailoring enzymes for specific applications through advanced screening methods, exploring sustainable production methods, and leveraging immobilization for process optimization (Madhavan et al. 2021). By addressing these challenges and capitalizing on the opportunities, the field of α-amylase can evolve and offer improved performance, stability, and cost-effectiveness

for a wide range of industrial applications. The following are the challenges in α-amylase:

Strain selection and culture-independent approaches

Selection of a suitable micro-organism with high α-amylase production capability is an essential yet challenging task (Elyasi et al. 2020). This is due to the significant variations that exist in enzyme production capabilities, yields, and productivities among different microbial species and strains. Screening a large pool of strains is often necessary to identify the most suitable one for efficient α-amylase production (Pranay et al. 2019) which makes it time, resource, and labor-intensive. Also, for scale-up and industrial applications, the desired strain should be fast-growing, easily culturable, and less prone to contamination. Recent research has emphasized the importance of using advanced screening methods, such as metagenomics and high-throughput screening techniques, to rapidly identify novel microbial strains with potential α-amylase production capabilities (Delavat et al. 2012; Motahar et al. 2021). For example, a cold-active α-amylase gene was found using the metagenomic approach, which showed a pH optimum of 8–9 and temperature optimum at 10–15 °C (Vester et al. 2015) which could be potentially used in the detergent industry. In another report, Motahar et al. (2020) screened sheep rumen metagee to find the thermostable and acidic amylolytic genes. In another interesting study, Nair et al. (2017) exploited marine sediments to discover potential microbes with substantial α-amylase activity with a view that the isolated enzyme may be able to withstand the typically harsh chemical conditions encountered in the industry. They created a metagenomic library in pUC19 from the marine sediments of the Arabian Sea obtained at a depth below 96 m, and the clone showed considerable amylolytic activity. The geothermal springs are believed to harbor an extensive array of undiscovered microorganisms. A thermostable amylase was isolated from the Odisha geothermal spring through metagenomics-based techniques, unveiling a gene comprising 1503 base pairs. This gene encodes a protein consisting of 469 amino acids, with a molecular weight of 53,895.05 Da and a pI of 7.78. Sequence analysis revealed a remarkable 98.95% identity with the α-amylase gene of *B. licheniformis* (Chauhan et al. 2023). The functional metagenomics approach in the Ethiopian soda lake led to the finding of various carbohydrate-degrading enzyme sequences (Jeilu et al. 2022). In cold environments, Proteobacteria and actinobacteria are widespread. Singh et al. (2022) in a similar study, reported careful examination of the metagenomic data from these environments to identify α-amylase enzymes with promising applications in the detergent industry.

One of the major challenges in the industry working under ambient operating conditions is that they are prone to contamination, resulting in product inconsistency and, at times, huge financial losses (Yassin et al. 2021). This is the primary reason that starch industries operate at high temperatures. But, at times, it leads to denaturation of the hydrolyzing enzymes. Thus, there is a need for a suitable strain/enzyme that can not only withstand severe operating conditions but also work effectively. To combat this issue, M/s Novozymes has developed an enzyme sold under the name of Termamyl Ultra from that can degrade cooked (gelatinized) starch with high efficiency at a temperature close to 90 °C with a working pH between 7 and 11 (https://www.ncbe.reading.ac.uk). Another challenge in strain selection for α-amylase production relates to regulatory and intellectual property considerations. Accessing and utilizing microbial strains may be restricted due to patent rights, biosafety regulations, or intellectual property conflicts. Recent discussions and developments in the field have emphasized the importance of open access to microbial resources, collaboration, and clear regulatory frameworks to overcome these challenges and promote innovation (O'Connor 2021).

Nutrient optimization

α-Amylase production relies on specific nutrients for bacterial growth and efficient enzyme synthesis. Identifying the optimal blend of carbon and nitrogen sources, trace elements, and other physical factors is not a simple task. Nutrient optimization studies are often required to maximize the yield of the enzyme (Kothakota, et al. 2021). Recent studies have investigated the effect of different carbon and nitrogen sources, on α-amylase production (Kamer et al. 2023) using statistical modeling and optimization strategies, such as Response Surface Methodology (RSM). By using the different techniques in RSM, such as the Box Behnken Design (BBD) and Central Composite Design (CCD), researchers have been able to study the intricate interactions between the different variables associated with α-amylase production and their influence on the response in the form of a non-linear regression model. By analyzing this model, researchers can identify optimal conditions that lead to the desired outcome, thereby improving efficiency, reducing costs, and enhancing product quality. A thermo-alkali stable α-amylase from *Bacillus* sp. was optimized using Box-Behnken design (BBD) by considering the physical factors such as pH, temperature, agitation speed, and incubation time. It was observed that the optimum conditions for α-amylase production were observed to be at more or less the central values of the variables. The variables pH, temperature, and incubation time showed both main effects as well as interaction effects with positive effects

on the system response, enhancing the α-amylase activity by ~ 1.5 folds (Khusro et al. 2017). In a similar study, optimizing α-amylase production in *Bacillus cereus* using a rotatable central composite design enhanced the enzyme activity by 3.9 fold (Ojha et al. 2020). The authors reported that the temperature and pH of the production medium significantly influenced the enzyme production. An increase in temperature beyond 30 °C or a reducing pH below 6.3 (which were identified as optimal conditions) reduced the enzymatic activity by 20–40%. Both the factors displayed main as well as interaction effects within the system. In another study, Saad et al. (2021) optimized α-amylase production from starch using CCD from *B. licheniformis* sp. It was observed that the enzyme productivity was enhanced by ~ 90% compared to the traditional OVAT optimization technique. The 3-D surface plots showed that starch concentration, pH, and incubation period displayed positive effects on the response (α-amylase activity) up to their optimal levels (10.5 g/L, 6.0, and 45 h, respectively), beyond which the values resulted in α-amylase repression. These studieselucidated that the micronutrients and growth factors either individually or with interaction effects play vital roles as cofactors or regulators of the enzyme (Fatoki et al. 2023). An inducer and signal molecules such as influencers can affect α-amylase production by regulating gene expression and signal transduction pathways. However, identifying suitable inducers and understanding their optimal concentrations and timing pose challenges. Recent approaches such as transcriptome and proteome analysis have explored the effects of different inducers and signal peptides on α-amylase production and investigated their underlying mechanisms (Huang et al. 2022).

Simair et al. (2017) stated that the high cost of fermentation media for amylase production is a major challenge to looking for agro-waste as a cheap substitute for fermentation media to reduce the overall cost. Substrate cost is a significant challenge in α-amylase production, accounting for 30–40% of the process (Niyomukiza et al. 2022). Cassava starch is cheaper than soluble starch and showed 170 times better results than soluble starch, which can be used for reducing fermentation costs (Ayansina et al. 2017) for large-scale industrial applications. Addressing the challenges in nutrient optimization for α-amylase production requires a multidisciplinary approach, encompassing metabolic engineering, systems biology, and process optimization. Recent advancements in next-generation sequencing technologies, computational modeling, and high-throughput screening methods have offered opportunities to gain a deeper understanding of nutrient requirements and develop tailored strategies for enhanced α-amylase production.

Oxygen transfer

Oxygen availability in liquid broth is a crucial factor influencing α-amylase production. Ensuring appropriate aeration and agitation to meet the oxygen requirements of the microbe is a must (Zhou et al. 2018). Microbial cells consume oxygen for different parallel pathways functioning inside cells, thus reducing the available dissolved oxygen in the fermentation broth, which may negatively impact α-amylase production. Recent research has focused on understanding and controlling the oxygen uptake rate through process optimization and oxygen supply strategies (Wang et al. 2020). Strategies such as intermittent aeration, oxygen feedback control, and metabolic engineering approaches have been explored to regulate oxygen uptake and improve enzyme production. In addition, the role of oxygen carriers such as n-dodecane and biosurfactants have been investigated to enhance oxygen solubility within the system (Wang et al. 2023). Mass transfer limitations can sometimes arise due to inadequate oxygen transfer from the gas phase to the bulk liquid and limitations in oxygen diffusion within the culture broth. The issues can be mitigated by optimizing bioreactor design, impeller configurations, and aeration strategies (Puiman et al. 2022). The use of oxygen-enriched air or pure oxygen, along with advanced oxygen delivery methods, has been observed to improve oxygen transfer efficiency considerably during α-amylase production (Mostafa et al. 2021).

Foam formation in bioreactors can also hinder oxygen transfer by reducing the effective gas–liquid contact area. The foam can accumulate at the liquid surface, impeding oxygen transfer and causing reactor instability. Blaga et al. (2022) investigated foam control strategies, including using antifoam agents, foam level control systems, and improved bioreactor designs, to minimize the adverse effects of foam on oxygen transfer and enhance α-amylase production. It has been well observed that oxygen transfer issues become more pronounced during the scale-up of α-amylase production processes. As the fermentation volume increases, maintaining an efficient oxygen supply becomes challenging due to increased agitation power requirements, oxygen demand, and limitations in oxygen supply. de Souza Vandenberghe et al. (2022) crucially looked into these facts and reported addressing the scale-up challenges by optimizing bioreactor geometries, aeration strategies, and process control parameters. In addition, Computational modeling and advanced monitoring techniques have been employed to study the fluid dynamics and the gas holdup within the system to understand and optimize the oxygen transfer at larger scales. Overcoming the challenges in α-amylase oxygen transfer requires a comprehensive approach that combines engineering, bioprocess optimization, and microbial physiology. Recent advancements in bioreactor design, oxygen delivery systems, and process control strategies offer opportunities for

improved oxygen transfer efficiency and enhanced α-amylase production.

Downstream processing

The purification of α-amylase from the fermentation broth can present certain challenges such as the presence of various cellular components and contaminants, low enzyme stability, and high viscosity of the fermentation broth which complicate and hinder the recovery process (Elyasi et al. 2020). For example, a high concentration of starch often leads to poor activity of α-amylase due to substrate inhibition (Božić et al. 2017). In such cases, recovery of the enzyme to achieve the desired purity in the industry becomes a demanding task (Wiltschi et al. 2020). Also, purification cost is an important consideration for the development of any α-amylase technology (Witazora et al. 2021). With the advent of technological advancements and the development of sophisticated purification methods, high-end purification of the enzyme may be possible but would also contribute significantly to the final production cost of the enzyme, increase the overall OPEX, and reduce the profit margin (Boodhoo et al. 2022).

Alternatively, to mitigate this issue, different purification techniques have been explored which include a combination of centrifugation, filtration, ultrafiltration, and chromatography to develop an efficient and cost-effective technology for enzyme recovery (Chen et al. 2022). In addition, chromatographic methods such as ion exchange chromatography, size exclusion chromatography, affinity chromatography, etc. have also been tried, to achieve high purity of α-amylase (Lim et al. 2020). In a more recent study, the use of novel materials, such as magnetic nanoparticles and molecularly imprinted polymers, has been explored for selective purification of α-amylase (Eivazzadeh-Keihan et al. 2021), with claims of 49.8% recovery.

Maintaining the stability of α-amylase during downstream processing is extremely crucial to preserve its activity and prolong its shelf life. However, α-amylase is susceptible to denaturation and degradation under certain processing conditions, such as high temperatures and extreme pH. Developing stabilization strategies, such as immobilization, encapsulation, formulation with stabilizing agents, and the use of additives and surfactants have been observed to considerably enhance enzyme stability and resistance to proteases (Li et al. 2022). In general, the recovery of α-amylase is cost-intensive due to the requirement for specialized equipment, consumables, and purification steps. The development of cost-effective downstream processing strategies is therefore extremely essential to make α-amylase production economically viable. Recent studies have explored process optimization, such as process intensification, process integration, and the use of novel separation techniques to reduce the overall cost of downstream processing (Meyer et al. 2021). Also, the utilization of renewable and low-cost raw materials for enzyme recovery and purification through innovative approaches needs to be investigated to obtain purity high-purity enzymes with substantial activities (Raina et al. 2022).

Scale-up considerations

Scaling up of α-amylase production presents significant challenges as it is extremely difficult to assess the most critical factors influencing the scale-up during fermentation due to a drastic change in environment (Crater and Lievense 2018). The transition from laboratory-scale to commercial-scale production requires careful consideration of several factors to ensure efficient and cost-effective processing for achieving the desired enzyme production levels (Deljou et al. 2018). Choosing the appropriate bioreactor design and mode of operation is of paramount importance. For example, impeller flooding is a condition that is often observed during α-amylase production, where the air stream along the stirrer shaft increases, which leads to poor mixing and low oxygen diffusion inside the medium (Mostafa et al. 2021). This typically occurs in industrial bioreactors where the pumping speed of the impellor is lower than the gas volume being introduced in the system. This issue can be mitigated by designing a broad blade impeller (within the bioreactor system) with a higher aeration number without compromising the aeration rates. This can efficiently prevent the microbial cells from settling down and ensure intimate contact between the air and the liquid medium with air dispersion and enhanced oxygen transfer rates. Besides oxygen transfer, other variables such as mixing efficiency and heat transfer effects must be carefully considered to ensure optimal growth and productivity of the microbial culture producing α-amylase. A few literature reports have even suggested the use of multiple bioreactors as a scale-up strategy for large-scale α-amylase production (Balakrishnan et al. 2021). Scaling up the downstream processing steps, such as harvesting, purification, and processing, is a complex task. Efficient separation and purification techniques need to be developed to handle larger volumes of the fermentation broth which requires both specialized facilities and skilled manpower. Additionally, optimizing the purification steps to achieve consistent enzyme quality is critical for successful scale-up. Robust quality control measures, including enzyme activity assays, purity analysis, and stability assessments, must be established and validated to ensure the product's quality meets the required specifications. Also, scaling up α-amylase production must consider cost optimization. Factors such as raw material sourcing, energy consumption, and process efficiency need to be carefully evaluated to achieve cost-effective production at a larger scale. Process

modifications and optimization can help reduce overall production costs without compromising the quality and activity of the enzyme. Successful scale-up of α-amylase production requires a comprehensive understanding of the fermentation process, efficient bioreactor design, robust downstream processing techniques, rigorous quality control, and careful cost optimization. Collaborative efforts between scientists, engineers, and industry experts are essential to overcome these challenges and ensure a smooth transition from laboratory-scale to commercial-scale production (Crater and Lievense 2018).

Strategy for α-amylase improvement

Although α-amylase has widespread applications occupying most of the world's enzyme market, it is still far from the ideal enzyme. The extremely high temperatures in the starch conversion industry limit the application of wild-type α-amylase (Gangadharan et al. 2020). Also, the oxidative environment of the detergent industry denatures the natural enzyme (Lim and Oslan (2021; Samanta 2022). Likewise, wild type α-amylase does not function well in an acidic environment; hence, raw juice clarification in the beverage industry is challenging (Liu et al. 2017; Bamigboye et al. 2022). Conventionally, it requires increasing the pH of the juice for optimum functioning of wild α-amylases, which ultimately leads to a change in the physicochemical characteristics of natural raw juice.

Additionally, the essential requirement of calcium for the functioning of the α-amylase typically restricts its action in a Calcium-deficient environment (Marengo et al. 2022). To overcome these limitations, extensive research has been focused on modifying the existing amylases and improving their activity/stability for effective functioning per the industrial requirements (Elyasi et al. 2020). As an option, chemical methods for amylase improvement have been tried. Yandri et al. (2012) attempted a chemical modification of *Bacillus subtilis* derived α-amylase using citraconic anhydride, which enhanced the thermal stability in contrast to the native enzyme. Similarly, Abdella et al. (2020) reported immobilization on chitosan magnetic nano-particles through physical adsorption and covalent binding, which improved its activity by ~ 2.3-fold (Abdella et al. 2020). Although initial observations suggest that the chemical treatments enhance the enzyme properties, in the long run, they have not been effective. Also, in some instances, the chemicals applied have been reported to threaten the environment and are ill-advised for large-scale applications (Li et al. 2022). Hence, protein engineering is the most preferred choice to generate more active and stable mutants in an eco-friendly manner compared to the chemical process. Genetic transformation can be done in two significant ways, namely, a) Directed evolution and b) Site-directed mutagenesis (Rabbani et al. 2011).

Directed evolution

The directed evolution method involves productive protein-sequence substitutions through mutagenesis followed by natural selection for generating a library of enzyme variants (Wang et al. 2021). Ruan et al. (2022) created a random mutation library of maltogenic amylase from *B. licheniformis* R-53 using error-prone PCR and successfully selected mutations with enhanced activity and thermal stability. The mutant V296F/K418I showed 2.16 times higher specific activity compared to the native one with optimum temperature enhanced by 5 °C from 60 °C. The authors observed that apart from improvement in dough recrystallization, the mutant α-amylase demonstrated reduced hardness and improved elasticity during bread storage compared to the native one. In another report, the pH stability of α-amylase from *B. licheniformis* was amplified by directed evolution with the help of error-prone PCR, resulting in mutant G81R. It was selected by high throughput screening, retaining 10% of its initial activity at pH 4.5 after 40 min in contrast to the native one, which was inactive in similar conditions. This enhanced stability might be due to increased electrostatic interaction, helix propensity, and hydrophilicity (Huang et al. 2019). Wang et al. (2012) developed a focused mutant library using a modified method, i.e., coevolving site saturation mutagenesis (CCSM) in directed evolution to improve the thermostability of α-amylase from *Bacillus subtilis CN7 (Amy7C)*. Co-evolution is the correlated variation of protein sites selected by various interactions like allosteric, hydrophobic, and synergistic effects. Correlated variation sites of protein were chosen as hotspots (H100, D144, T147, G89, D95, and N197) to prepare a focused mutant library, and a high throughput screening screened the hot spot residues through starch-iodine and DNS method. The novel mutation sites improved the thermal stability of wild-type amylase by 8 °C (Wang et al. 2012). The directed evolution method is in popular demand as it does not necessitate any requirement regarding the molecule's structure. Functional enzymes with desired properties can be easily attained by simply creating a mutant library of coding enzymes through random mutagenesis and selecting the desired mutant through high throughput screening, which can be extremely beneficial for industrial applications. For example, patent US10563186B2 (Andersen et al. 2017) describes random mutagenesis of a Termamyl-like α-amylase from M/s Novozymes. The mutation was targeted to enhance substrate specificity with enhanced thermal stability for application as a dishwashing detergent. The patent US10066222B2 (Callen et al. 2017) assigned to M/s BASF enzymes LLC, on the other hand, describes the involvement of random mutagenesis

for the improvement of α-amylase to remove starch from oil during the steeping process of corn. Another US patent US8828460B2 (Cherry et al. 2013) from M/s Novozymes describes the preparation of maltogenic α-amylases through random mutagenesis. The variants had altered pH enhanced thermostability, with an increased ability to reduce bread staling in the baking industry.

Site-directed mutagenesis

The site-directed mutagenesis method involves creating specific and intentional changes in the gene's DNA sequence of interest by targeted mutation of particular amino acids that alter or modify the intramolecular bonding within the protein or intermolecular interactions with a substrate (Fig. 3) Zhang et al. 2021a, b). Torktaz et al. (2018) carried out a series of point mutations in *Geobacillus stearothermophilus* to enhance its thermostability for potential industrial application. With the help of a prediction algorithm, 13 different sites were selected for point mutations, and the folding free energy was calculated after each modification. Although these amino acids are not part of the catalytic site, their alteration significantly affected the active site cavity by changes in substrate binding area and, thus, in binding affinity. The authors reported that four-point mutations D137V, G243V, E244I, and E244Y contributed significantly to making the *Geobacillus stearothermophilus* α-amylase more thermotolerant with higher ligand binding avidity. Industrial amylases,

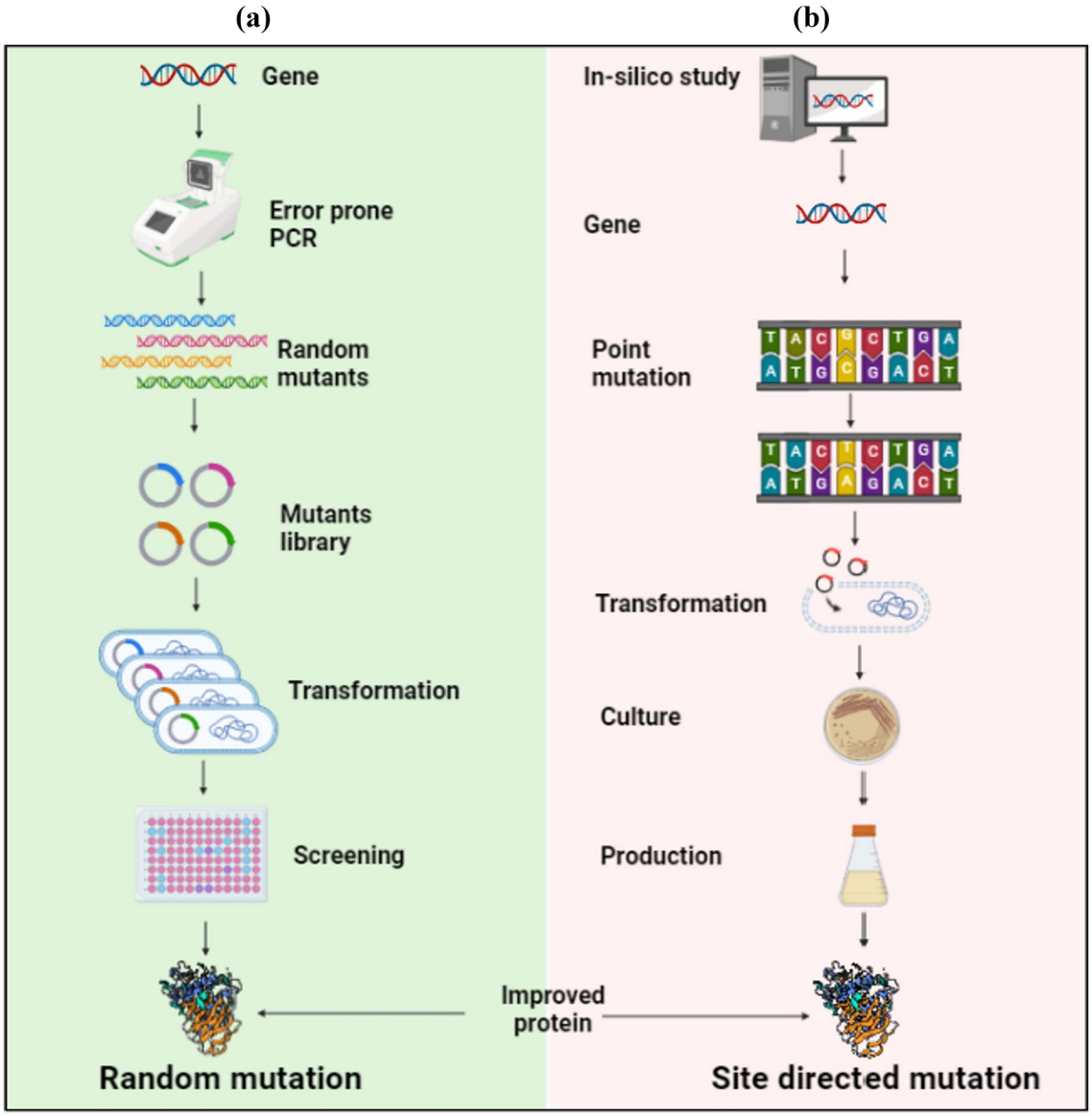

Fig. 3 Pictorial representation of **a** random and **b** site-directed mutation

generally produced by *Bacillus sp.,* have been extensively modified to incorporate desired properties such as secretion and enzymatic stability. For example, methionine residues within the protein have been determined as the most oxidation-prone residue and mutated to get oxidatively stabilized amylases for the detergent industry. Methionine mutation also enhanced the thermostability of enzymes. Declerck et al. (2003) aimed to enhance enzyme thermostability by targeting residues of hydrophobic packing of surface indentation of *B. licheniformis* α-amylase. It was reported that the point mutations aimed towards increasing thermostability by targeting residues of hydrophobic packing of surface indentation of *B. licheniformis* α-amylase. The mutations A209V and H133V enhanced the ligand binding and hydrophobicity responsible for protecting the secondary structure. Also, it was observed that N190F is the most thermo-stabilizing single mutation that could be achieved in *B. licheniformis*. The double mutants N190F and N265Y extended complex aromatic interaction with hydrogen bonds around the Ca-Na-Ca. This interaction reduced the enzyme's flexibility and stopped the escape of metal ions. On the contrary, the point mutation N192A made α-amylase more thermolabile by disrupting the hydrogen bonds, leading to helix to coil transition (Fort et al. 2021).

Literature reports suggest that the catalytic cleft in *B. licheniformis* contains conserved residues D206, E230, and D297 between domains A and B. Like *B. licheniformis*, other species have similar conserved residue profiles. H122 and H296 have been reported to assist in substrate binding. Pijning et al. (2021) Reported that alteration in conserved residues aspartate 206 and 297, glutamate 230, and histidine 122 and 296 in BLA enhanced the thermostability of the α-amylase. Deletion of Gly179 and Lys180 resulted in improved thermal stability due to reduced flexibility. Li et al. (2017a, b) carried out SDM of selected amino acid residues and showed that the mutation N188T and N188S enhanced the non-bond interactions, such as hydrogen bonds and salt bridges with the substrate. Also, the mutations A269K/S187D and A269K/S187D/N188T resulted in 25% less Km and 30% more K_{cat} than the wild type α-amylase. The triple mutant showed a half-life of 270 min (at 95 °C) at pH 5.5 and played an active role in the starch industry. α-Amylase obtained by the fusion of *Bacillus acidicola* with *Geobacillus thermoleovorans* had a melting temperature higher than the native one (Parashar and Satyanarayana 2016). Changes in N75, S76, and H77 of *Bacillus megaterium* resulted in better thermostability. α-Amylase from *B. licheniformis* (BLA) was mutated to increase its stability. It was observed that the Asparagines and Glutamines are prone to deamidation, which is accountable for thermal instability. Seven Asparagine residues were mutated at positions 172, 192, 190, 264, 265, 204, and 237. The authors reported that the Asparginine mutations at the positions enhanced the enzyme's stability. They further observed that N190F is the best mutation, even with different combinations, compared to other stabilizing mutations.

Bacillus licheniformis and *Bacillus amyloliquefaciens* are strains of industrial interest. Zhang et al. (2021a, b) focused on improving autolysis issues during amylase production. They found genes responsible for peptidoglycan hydrolase by transcriptomic profile during log and stationary phases. *lytd, lytE* and sig *D* were detected and mutated, resulting in a sharp reduction in hydrolyzed cells and a 48.4% increase in amylase activity. Deng et al. (2014) stated that the best mutated *AmyK* (The alkaline α-amylase gene AmyK) had a 5.4 °C increase in T_m and more than sixfold thermostability compared to the native type when four residues were simultaneously mutated. The hydrophobic and electrostatic interactions improved specific activity and thermostability (Li et al. 2017a, b). For example, Silano et al. (2020) reported an industrial α-amylase produced through genetic modification of a *B amyloliquefaciens* strain by M/s DuPont Danisco. The enzyme could be extensively used as a food amylase for the production of glucose syrups from starch (up to a recommended level of 45 mg TOS/kg) without any adverse toxicological effects. In a similar report, M/s Novozymes produced a recombinant maltogenic α-amylase using submerged fed-batch fermentation. The enzyme demonstrated very high activity (19,000 IU/g) and could be used in baking, brewing, and other cereal-based. Various examples of directed evolution and site-directed mutagenesis are given in Table 3.

A few industrial reports have demonstrated that SDM of α-amylases has significantly enhanced the functional properties of the enzyme for varied applications. For example, SDM of α-amylase in *B. licheniformis* (at M15L, from M/s GENENCOR) considerably enhanced the pH stability, oxidation, and heat stability, which are considered the most critical factors for application in the suitable industry. Another study from M/s Novozymes illustrates that alteration of the amino acid residue methionine (in *Bacillus* sp. at either 197th or 200th position) with a Leu, Ile, Asn, Ser, Gln, Asp or Glu enhanced the oxidation stability of the enzyme by many folds which can be used as a detergent additive. Likewise, US11248193B2 (Andersen and Fevre 2019) describes a SDM strategy for improvement of α-amylase for potential detergent applications in the industry.

Immobilization: an effective recycling strategy

Immobilization of α-amylase is a worthwhile strategy for improving stability alongside its reusability (Beltagy et al. 2022). The method employs retaining or binding enzymes over an insoluble matrix (through entrapment, adsorption, or covalent and cross-linking), enabling easy separation from the reaction mixture for successive recycling (Pervez et al.

Table 3 Genetic engineering strategies for activity and stability improvement of α-amylases

Sr. no.	Micro-organism	Mutation technique/mutated residues	Effect	Remarks	Application	References
	Random mutagenesis					
1.	*B. licheniformis R-53*	Error-prone PCR	A 2.16-fold increase in a specific activity	Increase in temperature stability by 5 °C. Mutated residues were V296F/ K418I	Bread baking industry	Ruan et al. (2022)
2.	*B. licheniformis*	Directed evolution	Mutant amylase retained 10% of the activity compared to optimum activity, whereas the wild type was inactive at pH 4.5	Mutation of G81R amylase resulted in a more hydrophilic membrane compared to wild-type	Starch processing industry	Huang et al. (2019)
3.	*A. flavus NSH9*	Ethidium bromide treatment	1.7-fold increase in activity	5 µg/mL EtBr gave maximum amylase activity	Starch processing industry	Ruaida (2021)
4.	*Aspergillus sp.*	Nitrous Acid (HNO_2)	Amylase activity increased by 60.85%	Deamination of adenine or cytosine	Waste degradation	Oshoma et al. (2022)
	Site-directed mutagenesis					
1.	*B. licheniformis*	S187D, N188T, A269K	Increased half-life ninefold at 95 °C	Reduction in the mobility of the loop containing these mutations	Starch processing industry	Li et al. (2017a, b)
2.	*B. subtilis CN7*	V260I	Increase in thermal stability by 7.1 °C	Mutation in a beta-strand of the TIM barrel	Starch processing industry	Wang et al. (2020)
3.	*E. sibiricum*	A109P/S130P/E176P	11.7-fold increase in half-life at 45 °C	Proline increases the rigidity of amylase	Detergent industry	Berlina et al. (2021)
4.	*B. licheniformis*	Q360C	3-fold increase in activity due to	Increased hydrophobic interaction at 70 °C for 30 min	Textile industry	Cui et al. (2022)
5.	*B. licheniformis*	H293R/H316R/H327R	31% of residual activity at 70 °C compared to inactive wild type	Increase in electrostatic interaction hydrogen bonding and hydrophilic interaction	Starch processing industry	Yihan et al. (2017)

2019). This is particularly important in large-scale production systems where production and processing cost is of paramount importance. The different methods for enzyme immobilization and their advantages are highlighted in Table 4.

For successful immobilization, it is necessary to choose a carrier with reasonable cost, and availability, and that has a good affinity towards the enzyme. The physicochemical parameters of the carrier including particle size, type of functional groups placed on the surface, surface area, and pore structure are governing factors for the immobilization step. Also, the support material should be insoluble and rigid. This reduces the chance of product inhibition by reducing non-specific interactions. A host of matrices have been investigated for α-amylase immobilization which may be organic, inorganic, or composite (Sharma et al. 2021). However, recently, hybrid matrices have become more popular owing to the advantages of high overall surface area, excellent ion exchange capability, environmentally friendly, and chemically inert with ease of activation. Yandri et al. (2022a, b) immobilized an α-amylase produced by *A. fumigatus* over a chitin-bentonite (CB) hybrid matrix and tested it for up to six cycles. The hybrid matrix displayed superior thermal stability in contrast to the classical matrices. The thermal inactivation rate constant (k_i) at 60 °C for the free and immobilized α-amylases were 0.0171 min^{-1} and 0.0045 min^{-1}, respectively. Reduced k_i indicated a decrease in denaturation due to higher flexibility in the water. The half-life ($t_{1/2}$) of the immobilized α-amylases was evaluated as 154 min, ~ 4-fold more elevated than the free enzyme (40.53 min). The free energy change due to denaturation (ΔG_i) was 104.47 kJ/mol for free enzyme, whereas 108.17 kJ/mol for the immobilized enzyme. The increased ΔG_i was due to increased folding conformations in the tertiary structure. The free α-amylases wasted ~ 72% of their original activity, whereas immobilized ones showed less than 30% loss. Immobilized α-amylase on CB hybrid can be used for up to six cycles by retaining 38% residual activity. Zhouquan et al. (2022) immobilized α-amylase on cellulose-chitosan hybrid gel macro sphere (CCMs) prepared by a sol–gel method having a high specific surface area (235.4–325.3 m^2/g), overcoming the limitation of the low specific surface area of cellulose. The Immobilized α-amylases displayed improved stability compared to free enzymes and reusability up to 10 cycles with 77.55% residual activity. The stability of α-amylase was improved by Karaca Açarı et al. (2022) due to the immobilization of carbon and graphene quantum dots (QDs). QD was prepared from *Hypericum perforatum* L flowers (QD-1) and *Hypericum capitatum* seeds (QD-2). Immobilization of α-amylase on QD-1 and QD-2 displayed activity efficiency of 71.15% and 81.51%. The difference in the activity efficiency was due to variation in the porosity of both Q.D.s. The free activation energy (E_a) was assessed to be 9.61 kJ/mol, 3.20 kJ/mol, and 4.81 kJ/mol for QD-1/α-amylase, QD-2/α-amylase, and free enzyme, respectively. Enhancement in the restriction of mobility of secondary structure resulted in more stability in the QD-1/amylase and QD-2/amylase in contrast to free α-amylase. Morais et al. (2013) immobilized α-amylase on *Luffa operculate* fibres. It was used in kitchen grease traps and showed 30% activity after 30 days. α-Amylase from *R. solani* AG-4 strain ZB-34 was immobilized on chitosan covalently. It retained 81% residual activity when used with Persil® detergent (for solid laundry). When tested for desizing ability, the immobilized enzyme demonstrated 31% desizing at 40 °C at pH 4.5 (Uzun and Akatin 2019).

Alternatively, immobilization of α-amylases on magnetic nanoparticles has been shown to improve the stability and activity of the enzyme under different operating conditions. In addition, these support materials have proven to offer extensive reusability by enabling simple recovery with negligible enzyme losses. Salem et al. (2021) immobilized α-amylase from *Bacillus subtilis* on Iron oxide magnetic nanoparticles (IO-MNP) by electrostatic bonds. The authors reported that the immobilized enzyme could work for ~ 15 cycles, retaining ~ 82% of the initial activity. In a similar study, Abouelkheir et al. (2023) created IOMNPs using *Bacillus subtilis* SE05, which showed very high α-amylase activity (592.92 U/mg) during starch hydrolysis for bio-ethanol production. The enzyme could be recycled up to 9 batches for starch hydrolysis with ~ 50% activity retained. Desai et al. (2021) alternatively immobilized α-amylases on graphene oxide-magnetite nanoparticles through covalent bonding. The immobilization enhanced the enzyme's half-life to ~ 20 h (from 13 h) at 50 °C with a marked increase in alkali tolerance. The immobilized enzyme could be used for up to 11 cycles and showed potential application in the production of high maltose containing syrup. Chitosan-coated Fe_3O_4 magnetic nanoparticles were used for α-amylase immobilization by Dhavaler et al. (2018). The immobilized enzyme showed no loss in activity up to 20 cycles and retained 66% activity after 3 weeks as compared to 18% activity of the free enzyme. α-Amylase immobilized on Magnesium ferrite nanoparticles functionalized with silane displayed catalytic activity even after 12 cycles of reaction (Rana et al. 2022). A more recent report by Hallol et al. (2022) produced chitosan-loaded barium ferrite nano-particles (CLBFNPs). α-Amylase derived from soil isolates was immobilized on them. The specific activity of nano-particle bound α-amylases was increased to 246.85 U/mg in contrast to the free enzyme (177.12 U/mg). The immobilized enzyme retained more than 90% activity even after five recycles. The authors concluded that the magnetic nano-particles were suitable enzyme carriers owing to their high surface area (SA) to volume (V) ratio, super magnetic characteristics, and biocompatibility. The outcome of immobilization has pointed out that the activity and the stability of

Table 4 Different techqniques and support systems for α-amylase immobilization

Approach	Method	Principle	Remarks	Drawback	Support	Activity	Recyclability	Application	References
Physical	Adsorption	Physical interactions occur between the enzyme and carrier without altering the native structure of the enzyme	Simple, cost-effective, and widely used method Immobilization is governed by particle size, functional group, surface area, and pore size of the support A high affinity of the support toward the enzyme is required	Non-specific interactions with other biomolecules may occur, which affects enzyme properties	Chitosan–Fe_3O_4 (CSM)	43.76% of free enzyme	10	*B subtilis* α-amylase immobilized on chitosan-magnetic nanoparticles enhanced dough-raising about 2.3-fold in baking	Abdella et al. (2020)
					Aniline formaldehyde condensate cross-linked polyaniline magnetic nanocomposite (AFC-CLPANIMg)	45% of free enzyme	12	NR	Augustine et al. (2023)
	Entrapment	The enzyme is trapped in synthetic or natural porous polymeric membranes	An affordable and fast method Requires mild conditions Protects the enzyme from mechanical shear, hydrophobic solvents, and gas bubbles	Diffusional substrate limitation causes decreased affinity towards substrate Loss in enzyme activity is also witnessed	Sodium alginate	56.7% of free enzyme	12	Encapsulated maltogenic amylase from *B stearothermophilus* maintained the bread's hardness and the crumb's structure even after four days of baking	Ningsih et al. (2020)

Table 4 (continued)

Approach	Method	Principle	Remarks	Drawback	Support	Activity	Recyclability	Application	References
Chemical	Cross-linking	Cross-linking can be achieved by mixing an enzyme with a polyionic synthetic or natural polymer and then cross-linking the polymer with multivalent cations in an ion-exchange reaction to form a lattice structure that traps the enzymes (ionotropic gelation)	Simple method with significant volumetric activity per biocatalyst mass, higher purity Low production costs and less contamination by the support A highly cross-linked gel can more effectively hold the enzyme	Low mechanical stability, intense activity, poor reproducibility, and difficulties with handling	Duolite® A-568	62.10% of free enzyme	NR	Termamyl® (2X) immobilized in Duolite® A-568 resin showed a twofold increase in activity and was used in starch hydrolysis in the industry	Silva et al. (2023)
					Graphene oxide (GO)	82.6% of free enzyme	NR	NR	Zhang et al. (2020)
					Amidrazone acrylic fabric	53% of free enzyme	15	NR	Al-Najada et al. 2019
	Covalent attachment	This method involves forming a covalent bond between functional groups on the support surface and the functional groups belonging to the amino acid residues on the enzyme's surface	The covalent binding of an enzyme to carriers leads to stable linkages, thus inhibiting leakage completely	Rigorous preparation conditions and loss of enzyme activity due to reactions with toxic cross-linking reagents are always observed	Cellulose tosylate	9.9% of the free enzyme	8	Immobilized α amylase displayed high activity (165.24 IU/mL) with an ethanol yield of 0.12 g/g by *S cerevisiae*	Verma and Raghav (2022)
					Egg membrane	100% of free enzyme	15	NR	Narula (2023)

the α-amylase could be significantly enhanced through the different immobilization methods and demonstrate excellent advantages from an industrial viewpoint.

Summary and future recommendations

α-Amylases are pivotal enzymes and have prevailed in the chemical industry for many decades with applications in the food, detergent, textile, and paper industries. The ever-expanding global market with diverse application areas has prompted both the industry and academia in this field to isolate and develop new and improved versions of the enzyme. However, there exist a few technological and economic challenges in the successful scale-up of these laboratory-based processes to industrial/commercial production scales. Designing a suitable bioreactor by considering the fermentation broth's hydrodynamics, mixing effects, and heat and mass transfer effects can enable maximum enzyme yield by lowering process energy requirements. Similarly, the utilization of inexpensive and renewable substrates for enzyme production can lead to significant cost reduction. For example, Litchee seeds, which are primarily considered as waste (after the consumption of pulp) and generally thrown away, contain ~ 40% starch and pose an excellent renewable feedstock for the cultivation of α-amylases (Aqilah et al. 2023). Recently, we have been able to isolate 5 thermophilic novel bacterial strains from the soil below the Litchi tree with considerable α-amylase activity (*data unpublished*). Likewise, bakery wastes such as stale bread, bread rolls, and cookies, and vegetable and fruit wastes from local mandis/markets may also serve as a low-cost feedstock for the production of these enzymes.

One interesting application of the α-amylase could be in the field of bioremediation, particularly for the breakdown of petroleum-derived compounds (PDC). A few recent studies have shown that these enzymes (like bacterial P450s) are capable of degrading hydrocarbons such as n-alkanes (ranging from C10 to C14 carbon atoms) and in some cases low-density polyethylene (LDPE) (Karimi and Biria 2019). The addition of starch to the medium has been observed to accelerate the breakdown of PDCs by enabling some kind of surfactant activity, which reduces the issue of hydrocarbon mass transfer thereby enabling better uptake. This could have immense applications in the areas where oil spillage occurs or in the case of petroleum refineries where petroleum sludge is an issue with millions of dollars spent on its disposal. Site-specific mutagenesis may further enable the synthesis of tailored α-amylases with enhanced reactivity and durability which may be capable of degradation of polyaromatic hydrocarbons from PDCs. With the advent of new immobilization techniques using different carriers, the recyclability of the enzyme may be further improved for a stretch of 6–10 cycles without affecting its performance and stability. A perfect combination of the approaches would enable addressing the existing challenges for successfully deploying large-scale α-amylase technologies.

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Declarations

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