Purification and Characterisation of Thermostable α-Amylases from Microbial Sources

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α-Amylases (E.C 3.2.1.1) hydrolyse starch into smaller moieties such as maltose and glucose by breaking α-1,4-glycosidic linkages. The application of α-amylases in various industries has made the large-scale productions of these enzymes crucial. Thermostable α-amylase that catalyses starch degradation at the temperatures higher than 50 °C is favourable in harsh industrial applications. Due to ease in genetic manipulation and bulk production, this enzyme is most preferably produced by microorganisms. *Bacillus* sp. and *Escherichia coli* are commonly used microbial expression hosts for α-amylases (30 to 205 kDa in molecular weight). These amylases can be purified using ultrafiltration, salt precipitation, dialysis, and column chromatography. Recently, affinity column chromatography has shown the most promising result where the recovery rate was 38 to 60% and purification up to 13.2-fold. Microbial thermostable α-amylases have the optimum temperature and pH ranging from 50 °C to 100 °C and 5.0 to 10.5, respectively. These enzymes have high specificity towards potato starch, wheat starch, amylose, and amylopectin. EDTA (1 mM) gave the highest inhibitory effect (79%), but Ca^{2+} (5 mM) was the most effective co-factor with 155%. This review provides insight regarding thermostable α-amylases obtained from microbial sources for industrial applications.

Keywords: Purification; Characterisation; Thermostable; α-Amylase; Microorganism

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INTRODUCTION

The International Union of Biochemistry (IUB) establishes the categorisation of enzymes into six different classes, based on the mechanism of enzyme action. They are E.C 1 oxidoreductases, E.C 2 transferases, E.C 3 hydrolases, E.C 4 lyases, E.C 5 isomerases, and E.C 6 ligases. Amylases are enzymes that hydrolyse the glycosidic linkages in starch, and are thus categorised in the class of E.C 3 hydrolases. Amylases can be categorised into endo- and exo-amylases as well as 3 classes including α-, β-, and γ-amylases, catalysing the hydrolysis of α-1,4 and α-1,6-glycosidic bonds in starch, yielding a variety of disaccharides and monosaccharides.

Microorganisms, especially bacteria have proven to have short generation time and are one of the main sources of α-amylase. Thermophilic, mesophilic, and extremophilic

bacteria are good sources for thermostable α-amylases. These enzymes work optimally at extreme temperatures.

Saccharomyces cerevisiae (an edible yeast) as well as other fungi (*Aspergillus oryzae*) and bacteria (*Bacillus licheniformis* and *Bacillus stearothermophilus*) have been used to produce α-amylase especially in the food industry because of its "Generally Recognised as Safe" (GRAS) status honoured by the U.S. Food and Drug Administration (FDA) (Nevoigt 2008).

Many purification methods have also been established to purify α-amylases from microbial sources. The methods are ultrafiltration, salt precipitation, dialysis, and column chromatography. These methods give different yields and folds of purification. Characterisation of α-amylases from microbial sources, in terms of optimum temperature, optimum pH, thermostability, and pH stability has become important in determining their related applications as biocatalysts in many processes in industrial fields.

This review article provides an overview on microbial sources of thermostable α-amylases. Purification methods and characterisation of microbial extracellular thermostable α-amylases in terms of optimum temperature and pH, thermostability and pH stability, substrate specificity as well as effects of metal ions and inhibitors are also focused in this article. However, information on purification and characterisation of non-thermostable and non-microbial α-amylases are excluded.

AMYLASE AS BIOCATALYST

Amylases are biological catalysts or enzymes that catalyse the hydrolysis of starch; thus, they are categorised in the E.C 3 class of hydrolases. Amylases are classified into two groups, namely endo- and exo-amylases, depending on their mode of action. Endo-amylases randomly hydrolyse α-1,4-glycosidic linkages in the amylose or amylopectin of starch, yielding linear and branched oligosaccharides of different chain lengths. Exo-amylases only hydrolyse starch from the non-reducing end, forming short end products successively. Table 1 summarises the class, glycosidic bond specificity, mode of action, and products of amylases.

α-Amylase or glucan-1,4-α-glucanohydrolase (E.C 3.2.1.1) is a starch degrading, calcium metalloenzyme that hydrolyses starch into smaller moieties such as maltose and glucose (Singh *et al*. 2016). This endo-amylase catalyses the internal hydrolysis of α-D-1,4-glycosidic linkages in the starch to yield small molecular weight carbohydrate moieties of α-glucose, α-maltose, and α-limit dextrin (Singh and Guruprasad 2014). These hydrolysed products have their functional hydroxyl group (-OH) in the α-configuration; hence, this enzyme is named α-amylase.

β-Amylase (glucan-1,4-α-maltohydrolase; glycogenase; saccharogen amylase, E.C 3.2.1.2) is an exo-amylase that catalyses the hydrolysis of α-1,4-glycosidic linkages of starch, producing β-maltose and β-limit dextrin (Oktiarni *et al*. 2015). This exo-amylase is not synthesized by animal tissues but present in microorganisms contained in the digestive tract. γ-Amylase (glucan-1,4-α-glucosidase; amyloglucosidase; exo-1,4-α-glucosidase; glucohydrolase, E.C 3.2.1.3) can act as exo- or endo-amylase due to its ability to hydrolyse both α-1,4 and α-1,6-glycosidic linkages. However, γ-amylases have the optimum of pH 3 and are most efficient in acidic environments (Saini *et al*. 2017).

Table 1. Classification of Amylases (Singh *et al*. 2016)

Enzyme	Glycosidic bond specificity	Mode of Action	Products
α-amylase (Glucan-1,4-α-glucanohydrolase)	α-(1-4)- glucosyl	Endo oligosaccharides	Linear and branched
β-amylase (Glucan-1,4-α-maltohydrolase)	α-(1-4)- glucosyl	Exo Dextrin	Maltose and dextrin limit
γ-amylase (Exo-1,4-α-glucosidase; glucohydrolase)	α-(1-4)- glucosyl and Glucose α-(1- 6)-glucosyl	Exo/ Endo	Glucose

THERMOSTABLE α-AMYLASE

Thermostable α-amylases are relatively stable at high temperature. Most studies focus on the purification and characterisation of thermostable α-amylase secreted from bacteria, but not from fungi and yeast. Thermophilic bacteria are the most commonly used as α-amylase producers as they can survive in high temperature and produce enzymes having optimum temperatures higher than 50 °C.

Thermostability is crucial in industrial applications, as most processes are optimally performed at elevated temperature, where thermostable enzymes are not deactivated by heating the mixture to a certain temperature over a period due to their high denaturing temperature, unlike the mesophilic enzymes. Thermostable enzymes can be stored at room temperature, thus lowering the costs (Straathof and Adlercreutz 2014). There are three steps in starch hydrolysis, which are gelatinization, liquefaction, and saccharification. The gelatinization of starch is industrially carried out at 110 °C; thus thermophilic and extremophilic α-amylases are preferred for their efficiency and economical value (Zhang *et al*. 2017).

A novel α-amylase has been discovered in the strain of *Bacillus licheniformis* B4-423, exhibiting the optimal activity at 100 °C and pH 5.0. The enzyme is stable over a wide pH range (4.0 to 10.0) and exhibits more than 90% activity from 20 °C to 80 °C (Wu *et al*. 2018). Because of these favourable properties, the thermostable enzyme has been applied in many production processes such as wine brewing and fermentation, baking and food processing, the pulp and paper industry, and detergent treatment systems. Table 2 shows the optimum temperature, thermostability, and potential industrial applications of microbial α-amylases.

Table 2. Microbial Thermostable α-Amylases and their Industrial Applications

Microorganisms	Optimum Temperature and Thermostability	Industrial Applications	References
Bacteria			
Bacillus sp. BCC 01-50	65 °C; 60-70 °C	Detergent, starch saccharification	Simair *et al*. 2017
Anoxybacillus sp. YIM 375	80 °C; 70-80 °C	Starch liquefaction, textile decolouration and biofuel	Zhang *et al*. 2016
Anoxybacillus thermarum A4 strain	70 °C; -	Detergent	Baltas *et al*. 2016
Fungi			
Talaromyces pinophilus 1-95	55 °C; -	Starch-to-ethanol conversion	Xian *et al*. 2015
Komagataella phaffii GS115	65 °C; 55-70 °C	Liquefaction and saccharification	Gandhi *et al*. 2015

MICROBIAL SOURCES OF THERMOSTABLE α-AMYLASE

α-Amylase can be extracted from many sources such as animals, plants, and microorganisms. It is preferred to be industrially extracted and purified from microorganisms, especially bacteria and fungi. Microbial α-amylase can be easily isolated and selected using substrate specificity, serial dilution, and extreme conditions such as temperature and extreme pH. The desired α-amylase properties for specific industrial applications can be designed and improved due to the advancement of genetic engineering and media optimization (Xie *et al*. 2014).

Gandhi *et al*. (2015) stated that the main reasons for selecting microorganisms as sources of enzymes are the physiologically and physicochemically controlled access of microorganisms, higher product yield than other sources, convenient and easy recovery in downstream processes, and cost benefits in processing. Moreover, having microorganisms as expression systems of α-amylase is beneficial because of inexpensive media, great adaptability, not affected by seasonal fluctuations, more stability, and catalytic variation compared with other sources (Borrelli and Trono 2015).

Fungus is a preferred source compared with other microbial sources because fungal α-amylases have more accepted GRAS status (Gupta *et al*. 2003). Espargaró *et al*. (2012) also stated that bacteria such as *Escherichia coli* forms inclusion bodies (IBs) containing infectious prion if it is used as expression host for yeast proteins. As a eukaryotic expression host, yeast has its post-translational modifications (PTMs) more similar to higher level eukaryotes than bacteria (Ahmad *et al*. 2014).

Although it is beneficial as a eukaryotic expression system, there has not been much research performed to purify and characterise α-amylase from yeast. Gandhi *et al*. (2015) expressed and characterised recombinant SR74 recombinant α-amylase in *Komagataella phaffii* GS115 with the SR74 α-amylase gene transformed from *Geobacillus* sp. SR74 using the vector of pPICZαB/SR74 α-amylase. A higher yield of α-amylase from *K. phaffii* GS115 was recorded than in *E. coli* transformed by Kassaye (2009) using pET-32b/α-amylase as a vector. However, the expression of SR74 α-amylase in *K. phaffii* GS115 under the regulation of alcohol oxidase (AOX) promoter required high methanol concentration (1% (v/v) every 24 h) to induce the expression for 120 h. Thus, Nasir (2019) has cloned the gene into pFLDα expression vector under the control of formaldehyde dehydrogenase

(FLD1) promoter before transforming into a new yeast expression system, *i.e.*, *Meyerozyma guilliermondii* strain SO (Oslan *et al.* 2012). Optimization was performed and highest production was found after 12 h of cultivation without any inducers.

In a study concerning marine yeast isolation and industrial applications conducted by Zaky *et al*. (2014), enzymes from marine yeast (*Aureobasidium* sp. and *Pichia* sp.) are expected to have high salt tolerance, thermostability, barophilicity, and cold adaptivity as the yeasts live in high salinity environment. *M. guilliermondii* has been used as the research model organism named "flavinogenic yeasts", being capable of riboflavin over-synthesis during starvation for iron as well as the expression system of thermostable T1 lipase gene (Sibirny and Boretsky 2009; Oslan *et al*. 2015; Abu *et al*. 2017). Table 3 shows the sources of microbial α-amylase from different expression hosts and its mode of production.

Table 3. Sources of Microbial Thermostable α-Amylases

Expression Hosts	Genetic Sources	MW (kDa)	Production	References
Bacteria				
Anoxybacillus flavithermus	Novel	60	Extracellular	Agüloglu *et al*. 2014
Bacillus amyloliquefaciens BH072	Novel	~68	Extracellular	Du *et al*. 2018
Bacillus licheniformis AT70	Novel	85	Extracellular	Afrisham *et al*. 2016
Bacillus licheniformis B4-423	Novel	58	Extracellular	Wu *et al*. 2018
Bacillus methylotrophicus strain P11-2	Novel	44	Extracellular	Xie *et al*. 2014
Bacillus mojavensis SA	Novel	2 (> 200 kDa), 1 (30-40 kDa)	Extracellular	Hammami *et al*. 2018
Bacillus subtilis WB800 (ATCC 6633)	*Bacillus amyloliquefaciens* JH-06	~58	Extracellular	Chen *et al*. 2015
Escherichia coli BL21 (DE3)	*Bacillus subtilis* DR8806	76	Intracellular	Emtenani *et al*. 2015
Escherichia coli BL21	*Geobacillus* sp. 4j	62	Intracellular	Jiang *et al*. 2015
Geobacillus bacterium (K1C)	Novel	~59	Extracellular	Sudan *et al.* 2018
Fungi				
Aspergillus flavus NSH9	Novel	54	Extracellular	Karim *et al*. 2018
Aspergillus terreus NCFT 4269.10	Novel	15.3	Extracellular	Sethi *et al.* 2016a
Engyodontium album TISTR 3645	Novel	50	Extracellular	Ali *et al*. 2014
Komagataella phaffii	*Bacillus licheniformis*	58	Extracellular	Wang *et al*. 2015
Komagataella phaffii GS115	*Geobacillus stearothermophilus*	59	Extracellular	Gandhi *et al*. 2015
Komagataella phaffii GS115	*Aspergillus niger* CBS513.88	-	Extracellular	Wang *et al*. 2018
Talaromyces pinophilus 1-95	Novel	58	Extracellular	Xian *et al*. 2015
Trichoderma pseudokoningii	Novel	30	Extracellular	Abdulaal 2018

PURIFICATION OF MICROBIAL EXTRACELLULAR α-AMYLASE

Enzyme purification is crucial in obtaining a pure enzyme fraction from an impure enzyme crude extracted from available sources. Without enzyme purification, protein and enzyme activity cannot be characterised accurately due to the impurities in the crude extract, resulting in faulty information and data. The α-amylase gene must be overexpressed in the induction medium before purification is conducted. For every purification step performed, total protein content, total activity, specific enzyme activity, yield, and purification fold are calculated to indicate the effectiveness of the steps taken.

Ultrafiltration

Ultrafiltration is a widely used technique in concentrating and purifying proteins by their molecular weight (M_w). The most commonly used filtration membranes are of 10-kDa and 30-kDa molecular weight cut-off membranes. This technique is usually equipped before or after ammonium sulfate precipitation. Before being subjected to ammonium sulfate precipitation, the crude α-amylase expressed in *Bacillus subtilis* KIBGE HAS was filtrated twice against 100-kDa and 30-kDa molecular weight cut-off (MWCO) ultrafiltration membrane, whereby 3.4-fold purification and 20.61% yield recovery were obtained (Bano *et al.* 2011). While purifying α-amylase expressed in *Anoxybacillus* sp. YIM 342, the crude enzymes were subjected to an Amicon ultrafiltration cell with 3-kDa MWCO membrane. The yield of 82% and 1.33-fold purification were reported after ultrafiltration technique (Zhang *et al.* 2016).

An example of ultrafiltration after ammonium sulfate precipitation was performed by Baltas *et al.* (2016). The work involved purifying α-amylase expressed in a thermophilic *Anoxybacillus thermarum* A4 strain. After the precipitation of salt was suspended in MOPS buffer, the enzyme solution was washed and subjected to an Amicon ultrafiltration membrane with the MWCO of 30 kDa. A 75.2% yield recovery as well as 4.4-fold purification were reported with this ultrafiltration technique after performing salt precipitation (Baltas *et al.* 2016). Similarly, after performing salt precipitation, enzyme solution containing α-amylase expressed in *Talaromyces pinophilus* 1-95 was concentrated using a 10-kDa MWCO ultra-filtration membrane with 80.13% yield recovery and 1.77-fold purification being reported (Xian *et al.* 2015).

Salt Precipitation and Desalting

Salt precipitation is a technique to purify proteins from the crude enzymes by increasing the salt concentration gradually. The most common salt used in this method is ammonium sulfate, $(NH_3)_2SO_4$. Precipitation is started by salting in, *i.e.*, adding $(NH_3)_2SO_4$ salt into the crude enzymes slowly in a conical flask on a magnetic stirrer until all salt has dissolved completely.

While adding salt into solution, the increase in water surface tension increases the hydrophobic interaction between proteins and water, resulting in the folding of protein to decrease the contact surface area of the proteins to the solvent. Finally, the proteins are precipitated. The saturation of $(NH_3)_2SO_4$ used in precipitation is majorly dependent on the molecular weight of the proteins, where low molecular weight protein, *e.g.*, IL-1β (17.5 kDa), requires higher salt concentration compared with IgG (150 kDa) with the addition of 40% to 45% saturation $(NH_3)_2SO_4$ (Wingfield 2016).

Table 4. Salt Precipitation and Desalting of Microbial α-Amylases

Microbial Expression Hosts	Genetic sources	Salt concentration (%)	Desalting techniques	Results	References
Bacteria					
Anoxybacillus flavithermus	Novel	70	Dialysis (100 mM potassium phosphate buffer, pH 8.0)	1.2-fold purification with 81.7% yield	Agüloglu *et al*. 2014
Anoxybacillus sp. YIM 342	Novel	70	Dialysis (50 mM Tris-HCl buffer, pH 7.5)	1.33-fold purification with 82% yield	Zhang *et al.* 2016
Anoxybacillus thermarum A4	Novel	40-80	Dialysis (50 mM MOPS, pH 7.0)	4.4-fold purification with 75.2% yield	Baltas *et al.* 2016
Bacillus amyloliquefaciens BH072	Novel	70	Dialysis (Deionized water)	3.23-fold purification with 71.08% yield	Du *et al*. 2018
Bacillus licheniformis AZ2	Novel	60-90	Dialysis (50 mM Tris-HCl buffer, pH 7.0)	6.6-fold purification with 54% yield	Deljou and Arezi 2016
Bacillus methylotrophicus strain P11-2	Novel	80	Dialysis (20 mM Tris-HCl buffer, pH 7.5)	2.3-fold purification with 70.8% yield	Xie *et al*. 2014
Bacillus subtilis	Novel	40-60	Dialysis (100 mM phosphate buffer, pH 6.0)	4.75-fold purification with 16.66% yield	David *et al*. 2017
Streptomyces fragilis DA7-7	Novel	85	Dialysis (Glycine-NaOH buffer, pH 10)	7.06-fold purification with 69.94% yield	Nithya *et al*. 2017
Fungi					
Aspergillus flavus NSH9	Novel	80	Dialysis (50 mM phosphate buffer, pH 7.0)	1.84-fold purification with 30.69% yield	Karim *et al*. 2018
Aspergillus terreus NCFT 4269.10	Novel	40-80	Dialysis (100 mM phosphate buffer, pH 6.5)	2.305-fold purification with 36.95% yield	Sethi *et al*. 2016a
Talaromyces pinophilus 1-95	Novel	33	**HiPrep 16/10 desalting column** (20 mM sodium phosphate buffer, pH 6.5)	1.77-fold purification with 80.13% yield	Xian *et al*. 2015

While purifying a 44.0 kDa α-amylase from *B. methylotrophicus* P11-2, Xie *et al*. (2014) added solid $(NH_3)_2SO_4$ with 80% saturation under gentle stirring, and the suspension was centrifuged at 10,000 rpm for 30 min at 4 °C after incubation at 4 °C overnight. The percentage yield of α-amylase was 70.8% with a 2.3-fold purification and a specific activity of 57.6 U/mg. However, when Karim *et al*. (2018) were precipitating α-amylase expressed from *A. flavus* NSH9, the percentage yield of the enzyme was only 30.7% with 1.84 purification fold and a specific activity of 34.8 U/mg. It was interesting when Du *et al*. (2018) performed salt precipitation at 70% saturation on the crude enzyme containing α-amylase expressed from *Bacillus amyloliquefaciens* BH072, but the pellet

was dissolved and dialysed against sterile deionized water overnight. Such desalting technique was still able to achieve the purification fold of 3.23 as well as a yield of 71.1% which was high on average. This might be caused by the purified α-amylase exhibited its optimal activity at pH 7 (neutral). Even though salt precipitation cannot lead to highly purified protein, this technique can eliminate some unwanted protein and concentrate the sample.

Referring to Table 4, the precipitation and purification α-amylases from different microbial expression hosts were performed at the salt concentrations ranging from 33 to 90%, but the most common concentration used was 80%. However, the results reflected that α-amylases produced by bacteria required higher salt concentration compared to fungi. This phenomenon might be due to higher solubility and stronger interaction between bacterial α-amylases with water molecules compared to fungal α-amylases.

The most commonly used desalting technique is dialysis, depending on the buffers used to dissolve the pellet. Dialysis is the step following salt precipitation. It removes the salt after the pellet from post-precipitating centrifugation has been resuspended in buffer or to undergo buffer exchange when expression medium has different pH with purification column's pH. While Xian *et al*. (2015) were purifying α-amylase expressed from *T. pinophilus* 1-95, a 0.22 µm filter membrane (HiPrep 16/10 desalting column) was equipped to dialyse and filter out the eluted $(NH_3)_2SO_4$ after resuspending in 20 mM sodium phosphate buffer, pH 6.5. A higher yield of 80.1% was found compared with other fungal α-amylases desalted using dialysis, *e.g*., 30.69% for *Aspergillus flavus* NSH9 (Karim *et al*. 2018) and 36.95% for *Aspergillus terreus* NCFT 4269.10 (Sethi *et al* 2016a) (Table 4).

Column Chromatography

Ion-exchange chromatography

Ion-exchange column chromatography (IEX) is based on the ionic bonds between cations and anions. Duong-Ly and Gabelli (2014b) stated that IEX separates molecules by their surface charge, which deviates greatly between different proteins and enzymes. There are two distinct mechanisms in purification using IEX: competitive ionic binding and ion exclusion due to repulsion between similarly charged analyte ions and ions fixed on the column (Acikara 2013). To ensure a protein or an enzyme has a particular charge, it should be dissolved in buffers with pH lower or higher than its isoelectric point (pI). There are two phases involved in this chromatography namely mobile and stationary phases. The mobile phase is generally an aqueous buffer system that contained the crude enzyme. Nevertheless, the stationary phase is an inert organic matrix, which is chemically derived from ionisable functional groups that carries a displaceable oppositely charged ion (Cummins *et al*. 2010).

The common desorption (elution) method increases the concentration of a similarly charged species within the mobile phase, thus competing and eluting the enzyme of interest from the column. In the purification of α-amylase, the most commonly used ion-exchange column is **DEAE Sepharose**, with commercially available **HiTrap DEAE Sepharose FF** and **HiTrap Q Sepharose FF**. Referring to Table 5, all the resins used in IEX are anionic exchangers, indicating that all the tabulated α-amylases are negatively charged at the respective working pH from the buffers used. This could be explained based on the fact that the pH of buffers used are higher than the pI of these enzymes. Negatively charged α-amylases are able to bind to the positively charged resins and are eluted with different concentrations of chloride ion (Cl^-), which depends on its overall strength of negative charge. Other positively charged contaminants will flow out from the column without

binding to the resins, while other negatively charged contaminants will be separated from the α-amylases depending on the elution strength, thus in different elution fractions.

Referring to Table 5, the most commonly equipped columns in IEX are DEAE-Sephadex A-50 (Wu *et al*. 2017; David *et al*. 2017) and **Q-Sepharose** (Chen *et al*. 2015; Sudan *et al*. 2018). While Sudan *et al*. (2018) were purifying α-amylase from *Geobacillus* bacterium K1C, the dialysed enzyme sample was loaded on a **Q-Sepharose** column pre-equilibrated with 20 mM Tris-HCl buffer, pH 8.0 followed by elution with step gradient of 1 M NaCl. Although the purification fold had the range from 2.55 (Karim *et al*. 2018) to 34.33 (Xian *et al*. 2015), the yields (11.73 to 42.91%) were lower compared to other types of column chromatography. Table 5 also shows that KCl and NaCl have been used frequently during elution to desorb the enzyme of interest from the column matrix (stationary phase).

Table 5. Ion-exchange Column Chromatography of Microbial α-Amylases

Microbial expression hosts	Genetic sources	Ion-exchange chromatography methods and results		References
Bacteria				
Bacillus licheniformis B4-423	Novel	Column	**DEAE-Sephadex A-50**	Wu *et al*. (2017)
		Binding Buffer	50 mM Tris-HCl buffer (pH 7.0)	
		Elution Buffer	50 mM Tris-HCl buffer (pH 7.0) with 0-0.5 M NaCl	
		Results	8.34-fold purification, 42.91% yield	
Bacillus methylotrophicus P11-2	Novel	Column	**HiPrep DEAE FF (1 mL)**	Xie *et al*. 2014
		Binding Buffer	20 mM Tris-HCl buffer (pH 7.5)	
		Elution Buffer	20 mM Tris-HCl buffer (pH 7.5) with 0-1.0 M NaCl	
		Results	4.2-fold purification, 39.1% yield	
Bacillus subtilis	Novel	Column	**DEAE-Sephadex A-50**	David *et al*. (2017)
		Binding Buffer	10 mM Tris-HCl buffer (pH 8.0)	
		Elution Buffer	10 mM Tris-HCl buffer (pH 8.0) with NaCl (unknown concentration)	
		Results	9.31-fold purification, 12.61% yield	
Bacillus subtilis WB800 (ATCC 6633)	*Bacillus amyloliquefaciens*	Column	**Q-Sepharose**	Chen *et al*. 2015
		Binding Buffer	20 mM Tris-HCl buffer (pH 8.0)	
		Elution Buffer	20 mM Tris-HCl buffer (pH 8.0) with 0-0.5 M NaCl	
		Results	4.60-fold purification, 29.4% yield	
Escherichia coli BL21	*Laceyella* sp. DS3	Column	**DEAE-cellulose**	El-Sayed *et al*. (2019)
		Binding Buffer	100 mM phosphate buffer (pH 7.5)	
		Elution Buffer	100 mM phosphate buffer (pH 7.5) with 0-1 M KCl	
		Results	2.19-fold purification, 27.42% yield	

Geobacillus sp. K1C	Novel	Column	**Q-Sepharose**	Sudan *et al*. (2018)
		Binding Buffer	20 mM Tris-HCl buffer (pH 8.0)	
		Elution Buffer	20 mM Tris-HCl buffer (pH 8.0) with step gradient of 1 M NaCl	
		Results	6-purification fold, 22.1% yield	
Tepidimonas fonticaldi strain HB23	Novel	Column	**Mono-Q Sepharose**	Allala *et al*. (2019)
		Binding Buffer	25 mM acetate buffer (pH 6.5)	
		Elution Buffer	25 mM acetate buffer (pH 6.5) with linear gradient of 0-500 mM NaCl	
		Results	9.5-fold purification, 31% yield	
Fungi				
Aspergillus flavus NSH9	Novel	Column	**Amberlite IRA-400**	Karim *et al*. (2018)
		Binding Buffer	50 mM potassium phosphate buffer (pH 7.0)	
		Elution Buffer	50 mM potassium phosphate buffer (pH 7.0) with linear gradient of 0-1 M NaCl	
		Results	2.55-fold purification, 11.73% yield	
Talaromyces pinophilus 1-95	Novel	Column	**HiPrep Q XL 16-10 Sepharose**	Xian *et al*. (2015)
		Binding Buffer	20 mM sodium phosphate buffer (pH 6.5)	
		Elution Buffer	20 mM sodium phosphate buffer (pH 6.5) with linear gradient of 0-1 M NaCl	
		Results	34.33-fold purification, 19.21% yield	
Trichoderma pseudokoningii	Novel	Column	**DEAE-Sepharose**	Abdulaal (2018)
		Binding Buffer	20 mM Tris-HCl buffer (pH 7.2)	
		Elution Buffer	20 mM Tris-HCl buffer (pH 7.2) with 0.2 M NaCl	
		Results	15.7-fold purification, 18% yield	

Size-exclusion chromatography

Size-exclusion chromatography (SEC) or gel-filtration chromatography are often used for enzyme purification. Proteins of varying sizes are separated by columns consisting of a matrix of beads, which contain sieves of a particular size. Larger molecules are eluted earlier than small compounds, as the beads have cross-linked polyacrylamide, agarose, and dextran, where smaller compounds enter the sieves in the matrix of the stationary phase (Duong-Ly and Gabelli 2014a). According to Giridhar *et al*. (2017), porosity, *i.e*., pore size, is an important parameter. Because SEC separates molecules according to their size in solution, the process occurs wholly within the pore volume, which should be as large as possible. Due to the porosity of SEC, larger components of the analyte will be sampled by larger pores and *vice versa*. Thus, the larger molecules elute from the column first and smaller components will elute later (Striegel 2017; Berg *et al.* 2002).

Referring to Table 6, the most frequently equipped SEC matrix is **Sephadex G-100**

(Chen *et al*. 2015; Baltas *et al*. 2016; Allala *et al*. 2019; El-Sayed *et al*. 2019). This matrix shown promising yields while purifying α-amylases produced by *Anoxybacillus thermarum* A4 strain (74.6%), *Bacillus subtilis* WB800 (41.7%), *Escherichia coli* BL21 (76.53%), and *Tepidimonas fonticaldi* strain HB23 (41%). However, the highest purification fold was achieved by Sudan *et al*. (2018) at 49-fold, although its yield was the lowest at only 5.2%. Besides **Sephadex G-100**, **Superdex 75** has been used while purifying α-amylases produced by *Anoxybacillus* sp. YIM 342 (Zhang *et al*. 2016) and *Geobacillus* K1C (Sudan *et al*. 2018).

Zhang *et al*. (2016) performed gel filtration chromatography to achieve 32-fold increase in specific activity and a yield of about 10.4%. A **Hiprep QXL 26/60 column (Superdex 75)** was loaded with concentrated enzyme sample in 50 mM Tris-HCl buffer (pH 7.5) and eluted using the same buffer using an AKTA^{TM} time at a flow rate of 1 mL/min with 3.0 mL per fraction. In recent research, **Sephadex G-100** was loaded with enzyme solution before eluting with 0.1 M phosphate buffer (pH 7.5) while purifying recombinant α-amylase AmyLa from *Laceyella* sp. DS3 expressed in *E. coli* BL21 (El-Sayed *et al*. 2019). **Sephadex G-100** has a molecular weight fractionation range of 1-100 kDa, thus, the AmyLa from *Laceyella* sp. DS3 was shown to have 51.5 kDa from SDS-PAGE was small enough to enter the pores of the resin and was within the intermediate period of elution (El-Sayed *et al.* 2019).

SEC is also known as gel-filtration chromatography where the column resin acts as a filter to remove salts from the samples loaded. This desalting technique is usually performed as the finishing or polishing step to remove excessive salt after ammonium sulfate precipitation and ion-exchange chromatography, as high salt concentration may affect the downstream characterisation and crystallisation processes. After purifying bacterial α-amylase (44 kDa) expressed in *B. methylotrophicus* strain P11-2 using anionic exchanger **DEAE FF**, **Superdex 75 10/300GL** was used as a filter to remove NaCl from the active fractions of IEX (Xie *et al*. 2013). A similar desalting procedure was performed by Abdulaal (2018) while purifying fungal α-amylase (30 kDa) expressed in *Trichoderma pseudokoningii*, where **Sephacryl S-200** was equipped to filter off salt (0.2 M NaCl) from the active fraction of IEX (**DEAE-Sepharose**). To remove excessive ammonium sulfate salt from sample to be loaded into **Q-Sepharose**, **Sephadex G-100** was used, while Chen *et al.* (2015) was purifying bacterial α-amylase expressed in *B. subtilis* WB800 because unnecessary salt may affect the binding efficiency of IEX.

Table 6. Size-exclusion Chromatography of Various Microbial α-Amylases

Microbial expression hosts	Genetic sources	Size-exclusion chromatography methods and results		References
Bacteria				
Anoxybacillus sp. YIM 342	Novel	Column	**HiPrep QXL 26/60 (Superdex 75)**	Zhang *et al*. 2016
		Running Buffer	50 mM Tris-HCl buffer (pH 7.5)	
		Elution Buffer	50 mM Tris-HCl buffer (pH 7.5)	
		Flow Rate	1 mL/min, 3 mL per fraction	
		Results	32-fold purification, 10.41% yield	
Anoxybacillus	Novel	Column	**Sephadex G-75**	Acer *et al*.

sp. AH1		Running Buffer	100 mM Tris-HCl buffer (pH 7.0)	2016
		Elution Buffer	100 mM Tris-HCl buffer (pH 7.0)	
		Flow Rate	3 mL/min	
		Results	18-fold purification, 9% yield	
Anoxybacillus thermarum A4 strain	Novel	Column	**Sephadex G-100**	Baltas *et al*. 2016
		Running Buffer	50 mM MOPS (pH 7.0)	
		Elution Buffer	50 mM MOPS (pH 7.0)	
		Flow Rate	0.5 mL/min, 4 mL per fraction	
		Results	29.8-fold purification, 74.6% yield	
Bacillus methylotrophicus P11-2	Novel	Column	**Superdex 75 10/300 GL**	Xie *et al*. 2013
		Running Buffer	20 mM Tris-HCl buffer (pH 7.5)	
		Elution Buffer	20 mM Tris-HCl buffer (pH 7.5)	
		Flow Rate	0.5 mL/min, 1 mL per fraction	
		Results	13.1-fold purification, 7.0 % yield	
Bacillus subtilis WB800 (ATCC 6633)	*Bacillus amyloliquefaciens*	Column	**Sephadex G-100**	Chen *et al*. 2015
		Running Buffer	20 mM Tris-HCl buffer (pH 8.0)	
		Elution Buffer	20 mM Tris-HCl buffer (pH 8.0)	
		Flow Rate	-	
		Results	3.38-fold purification, 41.7% yield	
Escherrichia coli BL21	*Laceyella* sp. DS3	Column	**Sephadex G-100**	El-Sayed *et al*. 2019
		Running Buffer	100 mM phosphate buffer (pH 7.5)	
		Elution Buffer	100 mM phosphate buffer (pH 7.5)	
		Flow Rate	-	
		Results	1.82-fold purification, 76.53% yield	
Geobacillus sp. K1C	Novel	Column	**Superdex-75**	Sudan *et al*. 2018
		Running Buffer	50 mM sodium acetate buffer (pH 6.0)	
		Elution Buffer	50 mM sodium acetate buffer (pH 6.0)	
		Flow Rate	0.5 mL/min, 1 mL per fraction	
		Results	49-fold purification, 5.2% yield	
Streptomyces fragilis DA7-7	Novel	Column	**Superdex G-100**	Nithya *et al*. 2017
		Running Buffer	50 mM Tris-HCl buffer (pH 9)	
		Elution Buffer	50 mM Tris-HCl buffer (pH 9)	

		Flow Rate	-	
		Results	17.34-fold purification, 24.62% yield	
Tepidimonas fonticaldi strain HB23	Novel	Column	**Sephadex G-100**	Allala *et al*. 2019
		Running Buffer	50 mM HEPES buffer (pH 7.0)	
		Elution Buffer	50 mM HEPES buffer (pH 7.0)	
		Flow Rate	0.5 mL/min, 3 mL per fraction	
		Results	6-fold purification, 41% yield	
Fungi				
Trichoderma pseudokoningii	Novel	Column	**Sephacryl S-200**	Abdulaal 2018
		Running Buffer	20 mM Tris-HCl buffer (pH 7.2)	
		Elution Buffer	20 mM Tris-HCl buffer (pH 7.2)	
		Flow Rate	-	
		Results	15.7-fold purification, 18% yield	

Affinity column chromatography

Affinity column chromatography purifies proteins according to their specific affinity towards a ligand. Such chromatography is also known as immobilization, which is normally called immobilized metal affinity chromatography (IMAC). When the analyte molecules in the crude enzymes interact with the solid resin of IMAC, which has a covalent linkage with a polydentate metal-chelating group binding to a metal ion, *e.g.*, nickel (Ni^{2+}), surface-exposed amino acid residues of the enzyme of interest will exchange with the water molecule in the metal coordination site, thus the enzyme is immobilized (Chang *et al*. 2017).

While purifying thermostable α-amylase from *B. subtilis* DR8806 but expressed in *E. coli* BL21 (DE3), Emtenani *et al*. (2015) loaded the clear supernatant containing intracellular α-amylase through Ni^{2+}-NTA matrix for affinity binding, yielding 60% recovery. Likewise, Gandhi *et al*. (2015) used IMAC to purify the α-amylase expressed in fungus with polyhistidine tag on 5 mL **HiTrap IMAC FF**, fast flow column with AKTA purifier system, yielding 1.9-fold purification with 52.6% recovery. Table 7 summarises affinity chromatography used to purify various microbial α-amylases.

Table 7. Affinity Chromatography of Various Microbial α-Amylases

Microbial expression hosts	Genetic sources	Affinity chromatography methods and results		References
Bacteria				
Escherichia coli BL21 (DE3)	*Bacillus subtilis* DR8806 in pET28a (+)	Column	Ni^{2+}-NTA matrix	Emtenani *et al*. 2015
		Binding Buffer	50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole (pH 8.0)	
		Elution Buffer	50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole (pH 8.0)	
		Results	60% recovery	

Escherichia coli BL21	*Geobacillus* sp. 4j in pET28a (+)	Column	Ni^{2+}-NTA resin		Jiang *et al*. 2015
		Binding Buffer	50 mM NaH_2PO_4, 250 mM NaCl (pH 5.5)		
		Elution Buffer	50 mM NaH_2PO_4, 250 mM NaCl, 500 mM imidazole (pH 5.5)		
		Results	13.2-fold purification		
Fungi					
Komagataella phaffii GS115	*Geobacillus stearothermophilus* in pPICZαB/α-amylase	Column	**HiTrap IMAC FF**, fast flow column		Gandhi *et al*. 2015
		Binding Buffer	20 mM NaH_2PO_4, 500 mM NaCl, 10mM imidazole (pH 7.4)		
		Elution Buffer	20 mM NaH_2PO_4, 500 mM NaCl, 500 mM imidazole (pH 7.4)		
		Results	52.6% recovery, 1.9-fold purification		
Komagataella phaffii GS115	*Bacillus licheniformis* in pPIC9K	Column	2 mL Ni^{2+}-chelating		Wang *et al*. 2015
		Binding Buffer	50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole (pH 8.0)		
		Elution Buffer	50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole (pH 8.0)		
		Results	5-L bioreactor	2.31-fold purification	
			50-L bioreactor	2.62-fold purification	

CHARACTERISATION OF MICROBIAL EXTRACELLULAR α-AMYLASE

α-Amylase can be characterised in many respects such as the effects of temperature and pH, thermostability, pH stability, substrate specificity, effects of metal ions and chelating reagents, inhibitors and activators, and kinetics constants. The determination of optimum temperature and pH as well as the stabilities are crucial especially in identifying the most suitable microorganisms to be used in specific industrial production processes. In every characterisation, DNS method (Gandhi *et al*. 2015) is used to quantify the enzyme activity.

Optimum Temperature and pH

Characterisation of α-amylase in terms of optimum temperature and pH enables industrial processes utilizing these α-amylases to be performed at the optimal rate, thus maximizing their yield. Referring to Table 8, α-amylase produced by *Bacillus licheniformis* B4-423 (Wu *et al*. 2017) showed highest activity at 100 °C compared to the lowest optimum temperature exhibited by that from *Streptomyces fragilis* DA7-7 (Nithya *et al*. 2017) in terms of bacterial α-amylases. However, both fungal thermostable α-amylases expressed in *Aspergillus flavus* NSH9 (Karim *et al*. 2018) and *Trichoderma pseudokoningii* (Abdulaal 2018) exhibited the lowest optimum temperature at 50 ˚C, while α-amylase produced by *Komagataella phaffii* (Wang *et al*. 2015) showed the highest optimum temperature at 90 °C. Bacterial α-amylases have a wide range of optimum pH from pH 5.0

(Wu *et al*. 2017; Emtenani *et al*. 2015) to 10.5 (Baltas *et al*. 2016), while fungal α-amylases were shown to have their optimum pH ranging from pH 5.0 (Karim *et al.* 2018; Sethi *et al.* 2016b; Xian *et al.* 2015) to pH 9.0 (Ali *et al.* 2014).

The difference of optimum temperature between bacterial and fungal α-amylases could be due to the characteristics of the bacteria and fungus, which are the expression hosts of the enzymes. Thermophilic bacteria generally have higher resistance toward high temperature compared to thermophilic fungi; thus, the α-amylases expression in thermophilic bacteria will probably exhibit higher optimum temperature compared to those expressed in thermophilic fungi.

Table 8. Optimum Temperature and pH of Extracellular α-Amylase from Microorganisms

Microbial Expression Hosts	Genetic Sources	Optimum Temperature (°C)	Optimum pH (pH)	References
Bacteria				
Anoxybacillus flavithermus sp. nov. SO-19	Novel	70	6.0	Özdemir *et al*. 2016
Anoxybacillus flavithermus	Novel	55	7.0	Agüloglu *et al*. 2014
Anoxybacillus sp. YIM 342	Novel	80	9.0	Zhang *et al*. 2016
Anoxybacillus sp. AH1	Novel	60	7.0	Acer *et al*. 2016
Anoxybacillus thermarum A4 strain	Novel	70	5.5-10.5	Baltas *et al.* 2016
Bacillus amyloliquefaciens BH072	Novel	60	7.0	Du *et al*. 2018
Bacillus licheniformis B4-423	Novel	100	5.0	Wu *et al*. 2017
Bacillus licheniformis AT70	Novel	60	8.0	Afrisham *et al*. 2016
Bacillus licheniformis AZ2	Novel	80	7.0	Deljou and Arezi 2016
Bacillus methylotrophicus strain P11-2	Novel	70	7.0	Xie *et al*. 2014
Bacillus mojavensis SA	Novel	55	9.0	Hammami *et al*. 2018
Bacillus subtilis	Novel	60	7.0	David *et al*. 2017
Bacillus sp. BCC 01-50	Novel	65	9.0	Simair *et al*. 2017
Escherichia coli BL21 (DE3)	*Bacillus subtilis* DR8806	70	5.0	Emtenani *et al*. 2015
Escherichia coli BL21	*Geobacillus* sp. 4j	65	5.5	Jiang *et al*. 2015
Escherichia coli BL21	*Laceyella* sp. DS3	55 (Free and Immobilized)	6.0 (Free) 7.0	El-Sayed *et al*. 2019

			(Immobilized)	
Geobacillus sp. K1C	Novel	80	6.0	Sudan *et al*. 2018
Streptomyces fragilis DA7-7	Novel	50	6.0	Nithya *et al*. 2017
Tepidimonas fonticaldi strain HB23	Novel	80	8.0	Allala *et al*. 2019
Fungi				
Aspergillus flavus NSH9	Novel	50	5.0	Karim *et al*. 2018
Aspergillus terreus NCFT 4269.10	Novel	60	5.0	Sethi *et al*. 2016b
Engyodontium album TISTR 3645	Novel	60	9.0	Ali *et al*. 2014
Komagataella phaffii	*Bacillus licheniformis*	90	7.0	Wang *et al*. 2015
Komagataella phaffii GS115	*Geobacillus stearothermophilus*	65	7.0	Gandhi *et al*. 2015
Komagataella phaffii GS115	*Aspergillus niger* CBS513.88 (AmyM)	60 (AmyM)	5.0 (AmyM)	Wang *et al*. 2018
Talaromyces pinophilus 1-95	Novel	55	5.0	Xian *et al*. 2015
Trichoderma pseudokoningii	Novel	50	7.0	Abdulaal 2018

Thermostability and pH Stability

Thermostability and pH stability are important factors in industrially applied α-amylase because most of the industrial processes are performed at elevated temperature and non-neutral pH. Most studies on thermostability of α-amylase used a range near to its optimal temperature. While characterising α-amylase expressed from *Anoxybacillus* sp. YIM342, a maximum activity was observed at 80.0 °C; thus, the range of temperature was set from 70 °C to 90 °C. α-Amylase expressed from strain YIM342 had its half-life after 30 min incubation at 80 °C, remaining >49% of its activity, thus suitable to be used in starch saccharification process.

In terms of pH stability, the enzyme was found to retain more than 80% of its activity after incubation at pH 8.0 and pH 9.0 for 210 min. 45% of original activity was still retained by α-amylase from strain YIM342 after being pre-incubated at pH 10.0 for 210 min (Zhang *et al*. 2016). α-Amylase expressed from *Aspergillus flavus* NSH9 was found to be thermally stable at 50 °C, with 87% residual activity after incubation for 60 min. It was also observed that α-amylase from strain NSH9 was able to retain almost 100% of its original activity after incubation at pH 6.0 and pH 7.0 for 24 h (Karim *et al*. 2018).

Although characterisation of α-amylase in term of thermostability is important, the stability of enzymes while they are stored at 30 °C as well as refrigerated at 4 °C is also significant to be determined. A study by El-Sherbiny and El-Chaghaby (2012) showed that

recovery of α-amylase (expressed in *Bacillus* sp.) with glycerol as a carrier or stabilizer at the storage temperature of 4 °C (114%) was higher than the sample stored at 30 °C (103%). However, when there was only water as carrier without glycerol as the stabilizer, the α-amylase recovery at 4 °C (117%) was significantly higher than the sample stored at 30˚C (30.7%). These results had shown that the significance and importance to have α-amylase shipped with glycerol as stabilizer at around 4 °C as the ambient temperatures for each country can be varied at high levels of fluctuation (El-Sherbiny and El-Chaghaby 2012).

Substrate Specificity

The substrate specificity profile is crucial because it characterises and determines the kind of starch that is degraded most effectively and efficiently by α-amylase. In the recent research conducted by Allala *et al*. (2019), the α-amylase TfAmy48 from *Tepidimonas fonticaldi* strain HB23 had the highest relative activity towards soluble potato starch (100%) while the enzyme had no activity towards some of the starches such as native potato, maize, rice starches, CMC and α-cyclodextrin. However, Baltas *et al*. (2016) found that the partially purified α-amylase from *Anoxybacillus thermarum* A4 strain had its highest specificity towards amylose (113%) and subsequently to soluble potato starch (100%) and amylopectin (93%), while the enzyme showed no activity towards cellulose as well as β-cyclodextrin (Table 9).

Both profiles in Table 9 reflected the preference of α-amylases to catalyze the hydrolysis of α-D-1,4-glycosidic linkages present at higher percentage in amylopectin, amylose, as well as soluble starches. Having a spontaneous hydrolysis rate of approximately 2×10^{-15} s^{-1} at room temperature, the α-glycosidic bond is very stable (Wolfdenden *et al.* 1998). The α-retaining double displacement proposed by Koshland (1953) is the most generally accepted catalytic mechanisms of the α-amylase family.

Five conserved amino acid sequence regions can be identified in members of the α-amylase family, where the two most conserved catalytic residues are located at the active site (glutamic acid as acid or base catalyst, and an aspartate as nucleophile) (Van Der Maarel *et al.* 2002). The third conserved residue, which is the second aspartate, binds to second and third hydroxyl groups (OH-2 and OH-3) of the substrate via hydrogen bonds, distorting the substrate (Uitdehaag *et al.* 1999). The fourth conserved amino acid residues can be histidine, arginine, and tyrosine, playing roles in ensuring correct orientation of the substrate into the active site, proper orientation of the nucleophile, transition state stabilization, as well as the polarization of the electronic structure of the substrate (Nakamura *et al.* 1993; Lawson *et al.* 1994; Strokopytov *et al.* 1996; Uitdehaag *et al.* 1999). An additional fifth conserved region also contains an aspartate, which is a calcium ligand (Janecek 1992).

Apart from the difference in conserved amino acid sequences, domain organization in various enzymes in the α-amylase family also has an effect on its substrate specificity. α-Amylase (E.C. 3.2.1.1), having A-domain (a highly symmetrical fold of eight parallel β-strands arranged in a barrel encircled by eight α-helices), B-domain (protruding between β-sheet no. 3 and α-helix no. 3 and playing a role in substrate or Ca^{2+} binding), as well as C-domain (unknown function), is meant to have starch (amylose and amylopectin) as its main substrate (Van der Veen *et al.* 2002). Thus, both conserved amino acid sequence and domains of the enzymes may contribute to their specificity to the substrate even though they are all in the α-amylase family.

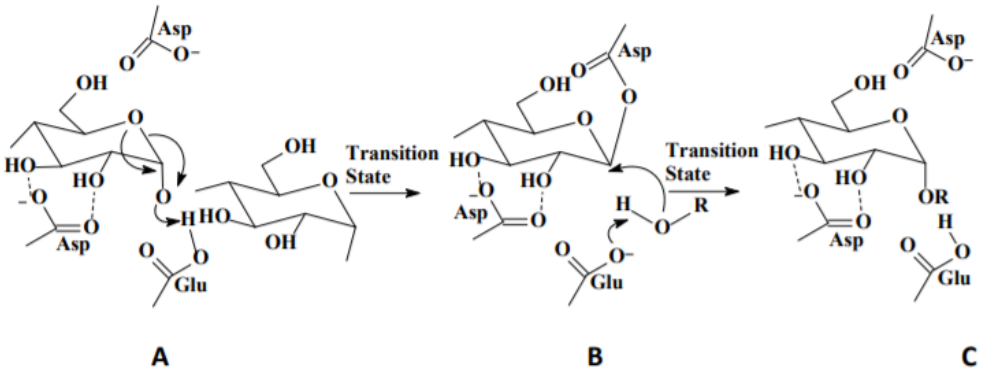

Fig. 1. The α-retaining double displacement method of α-amylase reaction mechanism (Van Der Maarel *et al.* 2002; Kumari *et al.* 2011)

Table 9. Substrate Specificity Profile of the Purified α-Amylases (Baltas *et al*. 2016; Allala *et al*. 2019)

Substrates		Relative Amylase Activity (%)	
		T. fonticaldi strain HB23 α-amylase TfAmy48	*A. thermarum* A4 strain α-amylase
Amylopectin		75	93
Amylose		85	113
Cellulose		-	0
CMC		0	-
Corn starch		-	≈87
Glycogen		-	50
Maize starch	Native	0	-
	Soluble	≈60	-
Potato starch	Native	0	-
	Soluble	≈100	100
Rice starch	Native	0	-
	Soluble	≈55	-
Wheat starch		79	≈89
α-Cyclodextrin		0	-
β-Cyclodextrin		-	0

Metal Ions and Inhibitors

Some metal ions of optimal concentration may act as cofactor in increasing the activity of α-amylase in degrading starch, while some reagents and inhibitors act to decrease its activity disregarding their concentration. Being a calcium metalloenzyme, α-amylase has elevated activity when calcium ion (Ca^{2+}) or salt ($CaCl_2$) is added in the reaction mixture; its activity increases by 8 ± 5%when 4 mM of Ca^{2+} is added to the reaction mixture containing α-amylase expressed from *Bacillus licheniformis* AT70 (Afrisham *et al*. 2016).

Allala *et al*. (2019) showed 55 ± 3.9% increased activity when 5 mM of Ca^{2+} was added to the reaction mixture with α-amylase purified from *T. fonticaldi* strain HB23. However, mercury ion (Hg^{2+}) showed an inhibitory effect on amylolytic activity (15 ± 3%) of α-amylase from strain AT70, which might be due to the non-specific binding and aggregation of the enzyme (Afrisham *et al*. 2016; Sethi *et al*. 2016b).

Referring to Table 10, Agüloglu *et al*. (2014) found the highest inhibitory effect when 1 mM EDTA (21%) and 10 mM EDTA (13%) were added separately to reaction

mixtures with α-amylase from *Anoxybacillus flavithermus*. This result also demonstrated that the chelating agent EDTA inactivated α-amylase, which is a metalloenzyme. When 10 mM EDTA was added to α-amylase from *Anoxybacillus* sp. AH1, the amylolytic activity dropped to 37%, with a 63% decrease in enzyme activity (Acer *et al*. 2016).

Table 10. α-Amylase Activity Remaining after Incubation for 30 min at 37 °C (Agüloglu *et al*. 2014)

Agents	Concentrations (mM)	Relative Enzyme Activity (%)
PMSF	1	91
	2	87
	4	74
	10	45
DTT	1	96
	2	87
	4	71
	10	73
β-Mercaptoethanol	1	98
	2	95
	4	92
	10	88
EDTA	1	21
	2	20
	4	18
	10	13

CONCLUSIONS

1. Affinity chromatography has shown the highest purification fold (1.72 to 13.2-fold) and recovery (38 to 60%) while purifying thermostable α-amylases in comparison to other purification methods such as ultrafiltration, salt precipitation, dialysis, and other means of column chromatography. An established purification method for microbial thermostable α-amylase is critical in fulfilling the demand of well-decontaminated and non-toxic enzymes in the industries.
2. Most studies have shown that microbial thermostable α-amylases have optimum temperature and pH values ranging from 50 °C to 100 °C and pH 5.0 to 10.5, respectively. Microbial thermostable α-amylase also shown to have high specificity towards soluble potato starch, wheat starch, amylose, and amylopectin. Both EDTA (1 mM) and mercury ion (Hg^{2+}) have been proven to strongly inhibit α-amylase activity, while calcium ions (Ca^{2+}) shown promising inducing effect (55%) on microbial α-amylase activity.
3. Purification and characterisation of α-amylase have been focused on enzymes from microbial sources (bacteria and fungi) as well as exhibiting thermostability. Such trends should be expected to have research contributing to an established purification method with more than 90% yield recovery and very high purification fold so that thermostable α-amylases can be purified thoroughly from microbial sources in large scale to ensure their safety to be used in various industrial applications.

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