A monograph on amylases from *Bacillus* spp.

Sailas Benjamin[*]**, R. B. Smitha, V. N. Jisha, S. Pradeep, S. Sajith, S. Sreedevi, Prakasan Priji, K. N. Unni, M. K. Sarath Josh**

Enzyme Technology Laboratory, Biotechnology Division, Department of Botany, University of Calicut, Kerala, India
Email: [*]sailasben@yahoo.co.in

ABSTRACT

Owing to the production of alpha, beta and gamma amylase subtypes; starch degrading microbes, especially bacteria have an invincible role in the food, fermentation, textile and paper industries. Of them, *α*-amylases from *Bacillus* spp. have contributed tremendous advancements in bio-industry, especially in starch, detergent and pharmaceutical arena. Though general reviews are seen in literature on amylases, no focused review is available yet solely on *α*-amylases produced by *Bacillus* spp. Hence, this focused review on *α*-amylases from the genus *Bacillus* is designed in such a way that it should give a vivid picture on most of the aspects on bacillial *α*-amylases in a handy module with an industrial perspective. With a short introduction on amylases in general, *α*-amylases from various species of *Bacillus* reviewed herein encompasses production of *α*-amylases by submerged and solid-state fermentations; nutrients and other factors required for maximizing production; immobilization strategies for whole cells or purified enzyme; an overview on the molecular weight of the enzyme; followed by distinct sections for purification, characterization, stability and crystal structure; and concluded with a section on industrial applications of the *α*-amylases from *Bacillus* spp.

Keywords: *Bacillus*; Amylases; Production; Fermentation; Crystal; Immobilization; Stability; Molecular Weight; Characteristics; Applications

1. INTRODUCTION

Enzymes are among the most important products obtained for human needs through microbial sources. A large number of processes in the areas of industrial, environmental and food biotechnology utilize enzymes at some stage or the other. Industrial enzyme market is an oligopoly with few strong players (amylases, proteases and lipases), and amylases are one among them. Amy-

lases are glycosidases which catalyse the hydrolysis of glycosidic linkage in starch to generate smaller sugars useful to bioprocess in bioindustry [1]. Although amylase can be derived from several sources—such as plants, animals and microorganisms—those obtained from microbial sources generally meet the industrial demands. The amylase family of enzymes has well been characterized through the study of various microorganisms. Amylases are traditionally produced by submerged or liquid fermentation (SmF) system. Of late, owing to its proven advantages, solid-state fermentation (SSF) advanced a lot to supersede the conventional SmF. SmF has been preferred for the production of industrially important enzymes because of the ease of handling and greater control of environmental factors such as temperature and pH [2]. Utilization of agro-industrial residues or effluents as medium for the fermentation received growing interests as they are inexpensive energy-rich sources and also eliminates large-scale accumulation of the biomass, and thus most of the commercial processes are based on SmF [3].

Classification of Amylases

Amylases are extracellular enzymes and classified into three subtypes, viz., *α*-amylase, *ß*-amylase and *γ*-amylase. Unlike *ß*-amylase, *α*- and *γ*-amylases are produced in animal systems abundantly, and all these three enzymes are produced by plants, yeast, fungi and bacteria. Four groups of starch converting enzymes have been identified viz., endoamylases, exoamylases, debranching enzymes and transferases [4]. Endoamylases cleave *α*-1,4 glycosidic bonds present in the amylose or amylopectin chain—the structural components of starch made of sugars—in a random fashion and *α*-amylase is a well known endoamylase. Exoamylases such as *ß*-amylase and glucoamylase cleave both *α*-1,4, and/or *α*-1,6 glycosidic bonds [4,5].

Alpha amylase (*EC* 3.2.1.1): is alternatively known as 1,4-*α*-D-glucan glucanohydrolase or glycogenase [6]. The *α*-amylases are calcium metalloenzymes, *i.e.*, the divalent calcium ion is invincible for its function [7]. By

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[*]Corresponding author.

acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates, ultimately yielding maltose and maltotriose from amylose or maltose, glucose and "limit dextrin" from amylopectin [8]. The hydrolytic products have α-configuration. As it can act anywhere on the substrate, α-amylase tends to be faster-acting than β-amylase [9].

Beta amylase (*EC* 3.2.1.2): is synonymous as 1,4-α-D-glucan maltohydrolase, glycogenase or saccharogen amylase synthesised by bacteria, fungi and plants [10]. Working from the non-reducing end, β-amylase catalyses the hydrolysis of the second α-1,4 glycosidic bond, cleaving off two glucose units (maltose) at a time [11]. During the ripening of fruit, β-amylase breaks starch into sugar, resulting in the sweet flavour of ripe fruits. β-amylase appears prior to germination, whereas α-amylase and proteases appear once germination has begun. Amylase from the cereal grains is the key source to the production of malt. Many microbes also produce amylase to degrade extracellular starches [12]. Animal tissues do not possess β-amylase, although it may be present in microorganisms contained within the digestive tract (probiotics).

Gamma amylase (*EC* 3.2.1.3): is also known as glucan 1,4-α-glucosidase, amyloglucosidase, exo-1,4-α-glucosidase, glucoamylase, lysosomal α-glucosidase or 1,4-α-D-glucan glucohydrolase [13]. In addition to cleaving the last α-(1-4)-glycosidic linkages at the non-reducing end of amylose and amylopectin yielding glucose, γ-amylase will also cleave α-(1-6)-glycosidic linkages [13]. Unlike the other forms of amylases, γ-amylase is best active in acidic environments [14].

2. α-AMYLASE PRODUCTION BY SUBMERGED FERMENTATION (SmF)

Conventionally industry prefers SmF for the large scale production of microbial enzymes due its easiness in controlling the process parameters, for which commercial media formulations are used. *Bacillus* spp. secrte a variety of enzymes including polysaccharases, proteases and nucleic acid hydolysing enzymes. Amylase from *B. subtilis* was considered as an industrial enzyme as early as 1917, but commercial production of this enzyme began in the late 1940s with the introduction of submerged culture or fermentation (SmF) borrowed from antibiotic industry [15]. Currently, a limited number of strains of selected species are used to prepare major seven enzymes including α-amylases on a large scale. Bacillial

Table 1. α-Amylase production of bacillial spp. in various media with or without starch supplement by SmF.

Bacillus sp.	Media with or without supplement	Yield (U/mL)	Reference
B. circulans	Corn starch and soluble starch	22	[17]
B. licheniformis		91.2	
B. megaterium		1.0	
B. subtilis		69.3	
B. subtilis	Soluble starch	13	[39]
Bacillus sp.	Soluble starch	75.7	[93]
Bacillu sp. KCA 102	Soluble starch	94	[4]
B. subtilis	LB agar medium	41.4	[94]
B. subtilis	Soluble starch	28	[68]
B. subtilis KCC 103	Sugarcane bagasse hydrolysate	144.5	[8]
B. halodurans	Soluble starch	1.8	[95]
B. licheniformis,	LB medium	1001	[96]
B. subtilis		2012	
Bacillus sp. Strain TSCVKK	Soluble starch	592	[97]
B. licheniformis	Soluble starch	260	[22]
B. licheniformis M27	Soluble starch	480	[98]
B. flavothermus	Lactose	28.6	[99]
B. subtilis JS 2004	Waste potato starch	72	[100]
Bacillis sp. Strain SMIA 2	Soluble starch	37	[101]

species like *B. amyloliquefaciens*, *B. licheniformis*, *B. stearothermophilus* and *B. subtilis* are the major producers of α-amylases [16]. **Table 1** illustrates media vs. enzyme production by various species or strains of *Bacillus*.

Bacillus spp. grows at different rate with specificity to different substrates in culture medium [17]. Complex or semisynthetic medium seems to be better for amylase production. Most of the media formulations use soluble starch as the principal carbon source in liquid media, whose percentage normally varies between 1 to 2 [18-20] which may be in combination with peptone [19] or yeast extract [19,20] or beef extract [21] or triptone [18]. Many authors evaluated efficacy of simple sugars such as lactose and glucose in combinations with nitrogen sources for amylase production using *Bacillus*. sp. As-1w [19] and *B. licheniformis* [22], and their efficiency was not promising in comparison to soluble starch or extracted starch from agricultural products like white corn [17] . Efficiency of *B. licheniformis* to thrive on starch from hordium, pearl millet, rice, corn, gram and wheat starch has been evaluated for the production of α-amylase [23], of which pearl millet starch (1.5%) significantly enhanced amylase production, which was also reconfirmed independently by another group [24].

Application of response surface methodology (RSM) for the optimization of medium and growth parameters found great advancement in reducing labour and time. RSM was efficiently used to study the cumulative interactive effect of the macronutrients (soybean meal, yeast extract and wheat bran) of the media and to optimize their concentrations to enhance the production (up to 25%) of maltooligosaccharide-forming amylase from *B. circulans* GRS 313 [25], or another combination comprising starch, glycerol, yeast extract and peptone from *Bacillus* sp. [26], wheat bran and groundnut oil cake mixture (1:1 ratio) cultural parameters for the cultivation of *B. amyloliquefaciens* in liquid state [27].

Like medium, controlled aeration also play a major role in maximizing the yield of α-amylase; and in general, lower agitation rate would be better for maximal α-amylase synthesis [28]. Mostly, maximum amylase production was noticed at 12 to 20 h of fermentation [4,18] or 24 h as in a batch process employing *B. licheniformis* [29]. Normally, *Bacillus* spp. prefer neutral or slightly alkaline or a range between 6.8 and 7.2 pH for amylase production at the initial stage of fermentation.

Various authors use different units of amylase activity for the interpretations of results. Predominantly U/mL is being used for SmF, but some authors used U/cfu (units per colony forming units) [17]. Established measure is the International Unit (IU) per mL culture medium in SmF, *i.e.,* micromoles of the product formed per minute [1]. For instance Ajayi and Fagade [17] demonstrated

different growth profiles and substrate specificites of five species of *Bacillus*; and that the amylase production values ranged from 0.22×10^2 U/cfu by *B. circulans* to 0.912×10^2 U/cfu by *B. licheniformis* from corn starch and 0.01×10^2 U/cfu by both *B. megaterium* and *B. licheniformis* to 0.693×10^2 U/cfu by *B. subtilis* from soluble starch. It seems very difficult to compare the activities between U/cfu and U/mL, hence it is always preferred to use the units of enzyme activity in U/mL when SmF is the fermentation strategy. When liquid media supplements (like extracts of agricultural residues) are used for SmF, it would be easy to compare if the values are given as liquid equivalents, *i.e.*, the control liquid medium, preferably a standard medium like LB or nutrient broth [1]. From SmF studies, it is evident that *B. licheniformis* and *B. subtilis* are the major producers of bacillial α-amylases. In general perspective and considering the thermostability, α-amylase from *B. licheniformis* is mostly preferred by the industry.

3. α-AMYLASE PRODUCTION BY SOLID-STATE FERMENTATION (SSF)

Current developments in biotechnology paved avenues for the novel applications of enzymes; accordingly the production strategies have been revolutionized. SmF has traditionally been used for the production of industrially important enzymes. In comparison, SSF with optimized production parameters seems to be better for enhanced amylase production [1]. Solid-state (substrate) fermentation (SSF) holds tremendous potential for the enhanced production of such industrially-significant enzymes. SSF has been defined as the fermentation process occurring in the absence or near-absence of free water [30] and it has become a very attractive alternative to SmF for specific applications due to the recent improvements in reactor designs [1]. SSF processes generally employ a natural and very cheap raw material as sole source of carbon and energy [30]. It can also be employed on inert material as solid matrix/support, which requires supplementing a nutrient solution containing necessary ingredients including carbon and nitrogen sources.

SSF has emerged as a potential technology for the production of microbial products such as feed, fuel, food, industrial chemicals and pharmaceutical products [31]. Its application in bioprocesses such as bioleaching, biobeneficiation, bioremediation, biopulping, etc. has offered several advantages such as higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, coupled with cultivation of microorganisms specialised for water-insoluble substrates and lower demand on sterility due to the low water activity used in SSF.

Agricultural residues offer a valuable substrate for amylase production by SSF (**Table 2**). As far as SSF is

Table 2. *α*-Amylase production of bacillial spp. on various solid media by SSF.

Bacillus sp.	Solid substrate	Yield (U/gds)	Reference
B. cereus	rice bran, wheat bran, sugarcane bagasse,	386	[34]
B. subtilis	black gram husk and green gram husk banana waste	7.27	[36]
B. licheniformis M27	Wheat bran	21,000	[102]
B. licheniformis	Potato peel and wheat bran	270,175	[33]
B. subtilis	Wheat bran	600,265	[33]
B. megaterium 16M	Wheat bran	30,000	[32]
B. subtilis	Wheat bran and rice husk	159,520 & 21,760	[37]
B. subtilis	Banana peel	9	[35]
Bacillus sp.	Wheat bran and lentil husk	172,800 & 216,000	[103]
Bacillus sp. PS-7	Wheat bran	464,000	[42]
B. cereus	Wheat bran and rice flake manufacturing waste	122	[104]
B. amyloliquefaciens	WB + Ground nut oil cake(1:1)	62,470	[105]
Bacillus sp.	Potato peel	270	[33]
B. subtilis SDA3,	Dehulled cooked soybean into soy-daddawa (a condiment)	4.0	[106]
B. circulans	Wheat bran	590	[107]
B. subtilis DSM 347,	Okpehe, a traditional fermented condiment in Nigeria	13	[39]

concerned, wheat bran seems to be the preferred substrate of interest for many investigators for *α*-amylase production [27,32-34]. Other agricultural residues like potato peel [33]; banana peel [35]; banana residue including whole plant after harvest [36]; residues generated during agricultural processing such as lentil husk [37]; rice bran, wheat bran, sugarcane bagasse, black gram husk, green gram husk [34], etc., seem to be efficient substrates. The success of SSF greatly depends on the appropriate incubation period, inoculum size, moisture level, suitable nitrogen source and pH requirements [1].

Interestingly, *in situ* fermentation using microbes bearing GRAS (generally regarded as safe) label to enhance the palatability and flavour of vegetables, pulses and other agricultural raw food stuffs is getting more attention in these days [38]. *B. subtilis* was found efficient to make Okpehe, a traditional fermented condiment in Nigeria; among other enzymes, amylase (13 U/mL) also contributed to the nutritional enrichment Okpehe [39]. *B. subtilis* was also used to convert sterile dehulled cooked soybean into soy-daddawa (a condiment) by the *α*-amylase (48 h, 35˚C). These studies show that *in situ* fermentation under controlled conditions for improving the organoleptic values of the raw food stuff, thereby increasing the price and shelf-life of the products which would in turn benefit the framers.

The activity of enzymes produced by SSF is normally expressed in U/gds (units per gram dry fermented substrate) [40]. However, when cross comparison of enzyme activities obtained from SmF and SSF is required, the authors may use confusing units of their choice, which may lead to false positive emphasis to the actual results.

4. OTHER NUTRIENTS FAVOURING *α*-AMYLASE PRODUCTION

Carbon (C) and nitrogen (N) sources, coupled with their proper blends are important parameters required for offering better growth of the microbes during fermentation. Optionally, multi-protein mineral media containing initial 2.5% or 3.5% concentration of starch and subsequent addition of 2% (at about 24 h after initiation of fermentation) was found better for the enhanced production of *α*-amylase [41]. Starch and tryptone were found ideal C and N sources, respectively for the production of amylase from *B. thermooleovorans*, but in a chemically defined medium consisting of glucose, riboflavin, cysteine, $MgSO_4$, K_2HPO_4 and NaCl, production increased by 2-fold [18]. Process parameters such as optimum substrate concentration, incubation period (42 h) and $CaCl_2$ (0.0275 M) requirements in the production medium emerge as crucial rate-limiting factors [27]. By SSF,

Sodhi *et al.* [42] found that *Bacillus* sp. PS-7 could produce a maximum of 464,000 U/gds (gram dry substrate) α-amylase on wheat bran supplemented with 1% glycerol, 1% soybean meal, 0.1% proline, 0.01% vitamin B-complex, 1% Tween 40, 1 mM $MgSO_4 \cdot 7H_2O$ at 48 h and $37°C$; in this complex medium, $CaCl_2$ was not specifically added. In fact, presence of $CaCl_2$ [20,36] and $MgSO_4$ [36] would boost the yield of α-amylase; also Ca, Mg and Na ions are implicated to play a major role in its yield and activity [1].

5. OTHER FACTORS CONTROLLING α-AMYLASE PRODUCTION

Enrichment of medium for fermentation is very much significant to maximize amylase production. Carbohydrates such as starch, dextrin, glycogen, cellobiose, maltohexaose, maltopentaose, maltotetraose and maltotriose were used for the enrichment culture of *B. stearothermophilus* in a complex medium containing beef extract or corn steep liquor [21]. Monosaccharides such as inositol and D-sorbitol have repressed amylase production, while organic and inorganic salts such as KCl, sodium malate and potassium succinate increased the yield than Na and K ions. Inclusion of aminoacids such as isoleucine, cysteine, phenylalanine and aspartic acid in the medium was also found vital for amylase production [21]. Detergents like Tween 80 and Triton X-100 may repress the enzyme production with increased biomass. *B. amyloliquefaciens* requires an optimum phosphate level in the medium for maximum α-amylase production by SmF [43].

As far as aerobic bacilli like *B. amyloliquefaciens* are concerned, aeration has an influential role in amylase production [44]. Production could be enhanced greatly by changing the shape of flask (baffled and non-baffled), working volume of fermentation media and shaking intensity [44]. The baffled flask (10% working volume) with 200 rpm, significantly increased the enzyme production. By increasing the aeration rate of 1 vv/m in 3 L stirred tank bioreactor, amylase activity was increased to 2-fold [44]. However, whenever a fermenter is involved for large scale production, near abolition of gas phase in it would be an essential factor, because it would increase the production (by 2.2 fold) with the reduction in optimal production time or gestation period [18]. Like aeration, optimum temperature, pH and $CaCl_2$ concentration also significantly increase amylase yield from species like *Bacillus* sp. AS-1 [19], *B. sphaericus* [45] and *B. amyloliquefaciens* [46]. Khan and Husaine [46] used 4% (w/v) sago pith residue (hampas) in 0.2 M citrate buffer at pH 6.0 at temperature $40°C$ and incubated for 6 h at 100 rpm with an additional supply of 1% soluble starch. It seems that, though raw starchy substrate would support amylase production by various *Bacillus* spp., addition of soluble starch (1% - 2%) in the medium will significantly boost the yield.

6. IMMOBILIZATION OF WHOLE CELL/AMYLASE

During the past few years, immobilized bacteria (cell factory/reactor) have been considered important for the production of industrially-significant enzymes [47]. Immobilized cells have many advantages over conventional fermentation, where freely suspended cells are used. These include: repeated or prolonged use of cells, easier downstream processing, reduced risk of contamination, continuous fermentation with less sophisticated reactors, *etc.* The immobilized cell reactor was observed to achieve larger volumetric productivities than either mode of stirred tank fermentations, but achieved an enzyme activity concentration lower than that of the batch stirred tank fermenter.

Immobilization was successfully accomplished for strains of *B. subtilis* in channeled porous alumina [48]; carrageenan gel [49] or in calcium alginate (2% sodium alginate and 3.5% $CaCl_2$, w/v) [50]; *B. licheniformis* in alginate or agar [51]; and *B. amyloliquefaciens* in macroreticular anionic exchange resin [52]. Amritkar *et al.* [53] incorporated polymeric substrates or substrate analogue during cross-linking of cellulose to prepare rigid, porous, cross-linked composite affinity matrices for the purification of α-amylase from immobilized *Bacillus* sp. B3. All these studies showed significantly higher amylase yields. For instance, strain of *B. licheniformis* showed more than 2 fold increase in enzyme yield upon immobilization in 4% alginate or agar; with optimal initial cell quantity as 0.6% - 3% in agar beads of 3 mm or 0.4% in Ca-alginate gel with 5 mm bead sizes [51].

In order to increase better stability and durability of the biocatalyst, immobilization and chemical modification strategies for the enzymes are being employed. α-amylase from *B. licheniformis* was immobilized on various carriers such as cross-linked cellulose matrix [54]; *B. circulans* in calcium alginate beads [25], amylases from *B. subtilis* on to coconut fibre [55] or of *Bacillus* sp. in to the magnetic poly glycidyl methacrylate beads [5]. Immobilization of enzymes generally stabilizes their structure and better adaptability to pH and temperature, coupled with increased half-life [56]. Immobilized amylases offer several advantages: they can be reused; the process can be operated continuously with better controls, easy separation of the products, simpler handling of the materials, alteration of activity and thermostability, effective reduction in process cost and long half-life of the enzyme. Covalent binding has been extensively used as a tool to

immobilize the enzymes. Immobilization will prolong the activity for several batches as in the casae of α-amylase from *B. licheniformis* [54]. Alternatively, addition of polyvinyl alcohol (0.25 wt%) [25] or $CaCl_2$ [56] on the reaction system significantly enhanced α-amylase activity.

7. PURIFICATION AND CHARACTERIZATION OF α-AMYLASE

Table 3 gives the summary of purification of amylase from various strains of *Bacillus* spp. Primary focus of all purification protocols is on ammonium sulphate fractionation and dialysis. Column chromatography employing sephadex [57] or sepharose [57,58] has been the major polymer matrices being used to purify the protein from partially purified ammonium sulphate fraction. Few others have used various affinity matrices for the purification [59,60].

Many authors showed that Ca^{2+} was essential for the stability and enhanced activity of α-amylases from *Bacillus* spp. [42,60,61]. Cations like Ca^{2+}, Na^+, Mg^{2+} showed stimulatory effect for amylase from *B. licheniformis*; while Ag^+, Hg^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+} and Al^{3+} showed inhibitory effect [60,61] and anions like azide, SO_3^{2-}, SO_4^{3-}, $S_2O_3^{2-}$, $MoSO_4^{2-}$ showed

excitant effect [60]. In fact, Cu^{2+} and Fe^{2+} (but not Ca^{2+}) were shown to have protective effects for amylase from *B. licheniformis* against EDTA-induced activity loss [60]. Contrary to the above reports, an alakaliphilic amylase from *Bacillus* sp. was strongly inhibited by Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+} and Cu^{2+}, whereas presence of Na^+, Co^{2+} and EDTA significantly enhanced the enzyme activity; and this feature is interesting to investigate at the molecular level [62]. Arikan [63] demonstrated that an amylase from *Bacillus* sp. A3-15 was inhibited by $ZnCl_2$, NaCl, $CaCl_2$, Na_2S, EDTA, urea and SDS and of *B. subtilis* was inhibited by the metal ions Cu^{2+}, Zn^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Cs^{2+}, Cd^{2+}, Fe^{2+}, Hg^{2+} and Ni^{2+} [64]. In general, if metal ion requirement is found essential for amylase activity, Ca^{2+} is the preferred ion for the stability and prolonged half-life of the enzyme [1].

Roles of temperature and pH on α-amylase activity seem to the interrelated. The temperature range for α-amylase activity varies from 40°C for an alkalophilic *Bacillus* sp. [62] to 95°C for amylases from *B. amyloliquefaciens* and *B. subtilis* [41]. However, most of the amylases are optimum active at around 65°C in a neutral pH environment [42,63,65]. It further shows that thermolabile amylases are mostly alkalophilic [62], while

Table 3. Activity and yield of bacillial α-amylases purified by various strategies.

Bacillus species	Purification strategy	Activity (U/mg)	Yield (%)	Purification (fold)	Reference
B. licheniformis	CMC column	882.7	42	211.67	[60]
Bacillus sp.	QAE column	18.5	2.4	-	[62]
B. licheniformis	Sephadex G-100, mono Q Sepharose anion exchange columns	178.5	15.9	3.08	[57]
B. amyloliquefaciens	Q-Sepharose & SP-Sepharose columns	31354	-	-	[6]
Bacillus sp. B3	AA-CELBEADS immobilisation	60.8	-	11.9	[53]
Bacillus sp. YX-1	DEAE-Sepharose Fast Flow & Sephadex G-75 columns	607	6.60	34	[108]
B. subtilis DM-03	DEAE Sephadex A-50, Sephadex G-50 & RP-HPLC columns,	1421	2.9	80.5	[109]
Geobacillus thermodenitrificans HRO-10	DEAE Sephadex A-50 and Superdex -200 columns.	684.3	11.5	13.6	[66]
B. licheniformis	Starch column	507	51	230	[59]
B. subtilis KCC-103	DEAE Sephadex A 50	482.4	45.2	19.9	[65]
B. kaustophilus	Metal-chelate column	39.7	49	11.4	[110]
B. subtilis	Sephadex G-75 column	916.7	23.4	187.1	[90]
Lactobacillus amylovorus	Sepharose 6B column	-	69	75	[58]
B. subtilis X-23	Q-Sepharose & Phenyl Sepharose columns	362	19.5	-	[77]

thermostable amylases are acidophilic [5,41]; and interestingly, all these amylases would best active at around pH 7 with good half-life.

Vmax and *Km* are coupled parameters which define the kinetic characteristics of an enzyme as a function of substrate concentration. These kinetic constants are critical to attempts to understand how enzymes work together to control cellular metabolism [30]. *Vmax* (moles/min) represent the rate of enzyme catalysis. *Km* (moles/L) value denotes the affinity of enzyme (here α-amylase) to the substrate (starch). Smaller values of *Km* indicate that the enzyme and substrate are tightly bound and form the enzyme-substrate complex more quickly, thus more activity. On the other hand, larger values of the *Km* constant indicate that the components are loosely bound and form the enzyme-substrate complex more slowly, indicatinbg low activity. Literature shows that *Km* of α-amylase from various species of *Bacillus* are normally expressed in mg/mL [60,62,65] with a value of 2.6 for *B. subtilis* [65], 3.05 for *Geobacillus thermodenitrificans* [66], 11.7 mg/mL for *B. acidocaldarius* strain RP1 [67], etc. However, *Vmax* has been represented in inconsistent units, which is difficult to correlate for a researcher; e.g., 1.08 mg/mL/min for *B. licheniformis* [60], 909 U/mg for *B. subtilis* [65], 0.051 μmol/min for a species of an alkalophilic *Bacillus* [62], 11.176 mg/mL/h for thermophilic *B. subtilis* strain [68], 600 milliunits/mg for *B. acidocaldarius* strain RP1 [67] or 7.35 U/mL for α-amylase secreted by *G. thermodenitrificans* [66]. From this, it is evident that expression of *Km* (mg/mL, though it should have expressed in moles/L) gives seemingly good clarity aand uniqueness of amylase activity, and it normally lies in a range between 1 - 3 mg/mL, but it may go up to a *Km* of 11.7 mg/mL as in *B. acidocaldarius* strain RP1 [67]. It is advisable that the authors should stick to the international units of expression, while reporting their findings to the scientific community.

8. MOLECULAR WEIGHT OF α-AMYLASE

In general, a survey shows that the molecular weight (MW) of α-amylase from *Bacillus* spp. varies between 50 and 60 kDa with some exceptions. Thermostable α-amylase from *B. licheniformis*, a monomeric enzyme with molecular mass of 55.2 kDa (483 amino acid residues) showed remarkable heat stability [69]. Liu *et al.* [70] found that the MW of a thermostable amylase from *B. licheniformis* was 53.13 kDa. The extracellular α-amylase produced by another strain (44MB82-A) of *B. licheniformis* was 58 kDa as judged by SDS-PAGE [71], but extremely smaller size of amylase (31 kDa) was demonstrated from another strain of *B. licheniformis* [29]. *B. acidocaldarius* strain agnano 101 produced an induc-

ible thermoacidophilic α-amylase, the purified amylase contained a single polypeptide chain of MW 68 kDa [67]. The apparent MW of the purified enzyme from *B. amyloliquefaciens* was 58 kDa as revealed by SDS-PAGE [27]. Amylase from *B. subtilis* strain BS5 showed 63 kDa [72], but another strain of *Bacillus* sp. produced an amylase whose MW was 126 kDa [73]—which may probably be a dimer.

The nucleotide sequence of the α-amylase gene from *B. stearothermophilus* and its flanking regions were determined by [74]. An open reading frame was found, comprising a total of 1647 base pairs (549 amino acids), starting from a GUG codon as methionine. It was shown by NH_2-terminal amino acid sequence analysis that the extracellular amylase consisted of 515 amino acid residues, which corresponded to a MW of 58.779 kDa. Thus, the NH_2-terminal portion of the gene encodes 34 amino acid residues as a signal peptide [74]. N-terminal signal peptides and C-terminal truncation have been characterized in many *Bacillus* spp. A novel liquefying α-amylase was found in cultures of an alkaliphilic *Bacillus* isolate, KSM-1378 corresponding to 516 amino acids that included a signal peptide of 31 amino acids. The calculated MW of the extracellular mature enzyme was 55.391 kDa [75]. C-terminal truncation has been observed on α-amylases of *B. subtilis* [76]. A *B. subtilis* amylase gene was cloned into a plasmid and expressed in *Escherichia coli*, the active protein was purified to apparent homogeneity [76]. Its MW (48 kDa) as estimated by SDS-PAGE was lower than the molecular mass values calculated from the derived amino acid sequences of the *B. subtilis* complete α-amylase (57.7 kDa). It indicates the removal of the N-terminal signal region from the functional enzymes. Complete (47 kDa) and truncated (67 kDa) isoforms of α-amylases from *B. subtilis* X-23 were purified, and the amino- and carboxyl-terminal amino acid sequences were determined [77]. The amino acid sequence deduced from the nucleotide sequence of the α-amylase gene indicated that truncated form was produced from complete isoform by truncation of the 186 amino acid residues at the carboxyl-terminal region.

9. STABILITY OF α-AMYLASE

Temperature and pH optima increase the stability of α-amylase. Amylase from *B. licheniformis* CUMC305 showed many interesting features. The purified enzyme showed maximal activity at 90°C and pH 9.0; and 91% of this activity remained at 100°C [60]. The stability of the amylase from *B. stearothermophilus* could be enhanced if liquified thick starch slurries (at 80°C and pH 6.9) were provided as feed/substrate in the presence of Ca^{2+} [21]. Modern molecular insights into the structure of

amylases and *in silico* approaches compelled the scientists for schimeric constructs based on the structural gene, the preferred choice for enhacing the stability of amylases. For instance, Suzuki *et al.* [78] showed that two regions in the amino acid sequence of *B. licheniformis* comprising Gln178 (region I) and the 255 to 270 residues (region II), respectively were shown to determine the thermostability of *B. licheniformis*; region I played the crucial role in determing the thermostability. Tomazic and Klibanov [16] reported that half-lives of *Bacillus* α-amylases at 90˚C and pH 6.5 greatly increased in the series from *B. amyloliquefaciens* to *B. stearothermophilus* to *B. licheniformis*. This stabilization is achieved by lowering the rate constant of monomolecular conformational scrambling, which was the cause of irreversible thermoinactivation of *B. amyloliquefaciens* and *B. stearothermophilus* α-amylases, so that deamidation of Asn/Gln residues for *B. licheniformis* emerged as the cause of inactivation. The extra thermostability of the thermophilic enzyme was found to be mainly due to additional salt bridges involving a few specific lysine residues (Lys-385 and Lys-88 and/or Lys-253).

10. CRYSTAL STRUCTURE OF α-AMYLASE

The α-amylase family is the largest sequence-based family of glycoside hydrolyses (GH13 family) and groups together a number of different enzyme activities and substrate specificities acting on α-glycosidic bonds [79]. α-amylases are classical calcium-containing enzymes which constitute a family of endo-amylases catalysing the cleavage of α-D-(1→4) glycosidic bonds in starch and related carbohydrates with retention of the α-anomeric configuration in the products [80]. Furthermore, these enzymes are used as targets for drug designing in attempts to treat diabetes, obesity and hyperlipemia [80]. It has long been known that α-amylases require calcium for their enzymatic activity.

Crystal structure of many α-amylase are known to date as shown in **Table 4**. It seems that α-amylases from *B. licheniformis* and *B. subtilis* have been the target of many investigators, owing to their predominant role in starch and drug industry. Typically, the α-amylases bear a (β/α) 8-barrel structure [81]. α-amylases with the (β/α) 8-barrel fold are involved in the catalysis of a wide variety of biochemical reactions [82]. The active sites of these enzymes are located on the C-terminal face of the central β-barrel [69]. Thermostable α-amylase from *B. licheniformis*, a monomeric enzyme with molecular mass of 55.2 kDa (483 amino acid residues), shows a remarkable heat stability [69]. Like other α-amylases, the polypeptide chain of *B. licheniformis* folds into three distinct domains. The first domain (domain A), consisting of 291 residues (from residue 3 to 103 and 207 to 396), forms a (β/α) 8-barrel structure. The second domain (domain B), consisting of residues 104 to 206, is inserted between the third β-strand and the third α-helix of domain A. The third C-terminal domain (domain C), consisting of residues 397 to 482, folds into an eight-stranded antiparallel β-barrel [69]. All known structures of holo α-amylases show a common calcium-binding site stabilising the interface between the highly homologous central A domain and the more variable B domain. As many crystal structures are known for α-amylase, their interrelatedness can easily be deduced by protein modeling [83].

Table 4. Strategies employed for the resolution of crystals from various α-amylases of *Bacillus* spp.

Sl. No	*Bacillus* sp.	Strategy	Resolution	Ref.
1	*Bacillus* sp. Strain KSM-K38	Molecular replacement method and refined to a crystallographic *R*-factor of 19.9% (*R*-free of 23.2%)	2.13 Å resolution	[111]
2	*B. subtilis*	Co-crystallised maltopentaose and acarbose, determined by multiple isomorphous replacement. Restrained crystallographic refinement has resulted in an *R*-factor of 19.8% in the 7.0 to 2.5 Å resolution range	2.5 Å resolution	[112]
3	*B. licheniformis*	Multiple isomorphous replacement in a crystal of space group $P4_32_12$ ($a = b = 119.6$ Å, $c = 85.4$ Å).	2.2 Å resolution	[113]
4	*B. licheniformis* 55,200 Da (483 amino acid residues)	Multiple isomorphous replacement method of X-ray crystallography	1.7 Å resolution	[69]
5	*B. stearothermophilus*		2.0 Å resolution	[114]
6	*B. halmapalus*	Complex with the (pseudo) tetrasaccharide inhibitor acarbose	2.1 Å resolution	[115]

11. APPLICATIONS OF *α*-AMYLASE

With the advent of new frontiers in biotechnology, the spectrum of the applications of amylase has been widened up to many other fields such as clinical, medicinal and analytical chemistries, as well as their widespread use in the industries such as textile, laundry, porcelain, detergents, paper, food, brewing, baking and distilling. If no suitable enzyme is available in nature, a suitable one will be designed to the choice of the industry as being done on *B. licheniformis*. The engineering works performed on *B. licheniformis* α-amylase from over the last decade provide a good example of the extent to which an enzyme can be remodeled in order to improve its natural performance and thus to fulfill industrial requirements. Contrary to expectations, the thermal resistance of this highly thermostable α-amylase is far from being maximised in the wild-type enzyme and there seems to exist many ways to increase the thermostability even further. A fair set of stabilising substitutions have already been found in *B. licheniformis* and many more may be identified in the future. Given the success achieved so far, it may even be possible to increase *B. licheniformis* thermostability beyond the most thermostable enzymes found in hyperthermophiles.

Table 5 shows that the bacillial α-amylases are mainly employed in food, detergent, textiles, paper and pharmaceutical industries. Enzymes are commonly used in the baking industry, as they can improve dough quality and texture and lengthen the shelf life of the final product. There is a little published information is available which highlights exposure of enzymes (other than fungal α-amylase) to the baking industry [84]. α-amylases have been used extensively in bread making to break down complex sugars such as starch (found in flour) into simple sugars [85]. Directed evolution coupled with a high-throughput robotic screen was employed to broaden the industrial use of the maltogenic α-amylase, Novamyl obtained from *Bacillus* sp. TS-25 [86]. Wild-type No

Table 5. Major applications of *α*-amylases obtained from *Bacillus* spp.

SI. No.	Industry	*Bacillus* sp.	Reference
1	Food industry	*Bacillus* sp. TS-25	[86]
		B. subtilis & *B. amyloliquefaciens*	[84]
		B. subtilis	[100]
		B. megaterium	[12]
		B. licheniformis	[89]
		Bacillus. sp. A3-15	[63]
		Bacillus. sp. strain SMIA-2	[101]
		B. cohnii	[116]
2	Detergent industry	*Bacillus* strains	[87]
		B. licheniformis	[89]
		B. horikoshii	[91]
		Bacllus sp. B$_3$	[117]
		Bacillus sp. A3-15	[63]
3	Textile industry	*B. licheniformis*	[89]
		Bacillus sp. B$_3$	[117]
4	Paper industry	*Bacillus* sp. NCIMB 40916	[91]
		Bacillus sp. B$_3$	[117]
		B. licheniformis	[118]
5	Pharmaceutical industry	*Bacillus* sp.	[119]
		B. licheniformis	[7]
		Bacillus sp.	[85]

vamyl is currently used in the baking industry as an anti-staling agent in breads baked at neutral or near neutral pH. However, the enzyme is rapidly inactivated during the baking process of bread made with low pH recipes and Novamyl thus has very limited beneficial effects for this particular application [86]. Alternatively, Rajagopalan and Krishnan [8] used glucose and maltose forming α-amylases from *B. subtilis* in alcohol fermentation and sugar syrup formulation; and also for the food processing by malto-oligosaccharide forming α-amylases.

The alkaline enzymes used in modern detergents are protease, cellulase, α-amylase, lipase, and mannanase [87]. Like proteases, alkaline amylases play crucial role in detergents designed for washing machines [62,88]. Large quantities of alkaline enzymes are used in the detergent industry, and they have widely been incorporated into heavy-duty laundry and automatic dishwashing detergents. Though traditionally prepared from *B. amyloliquefaciens*, the amylase from *B. licheniformis* is getting growing market shares because of its greater thermostability [57].

The major uses of amylases extend to textile and paper industries also. For a number a of years, α-amylases have been used for a variety of purposes; the most important of which are starch liquefaction, textile desizing, and starch modification in the paper and pulp industry [89]. One of its major uses is for the liquefaction of starch (reduction of high molecular mass to about 10 or 11 residues) in the starch processing and related industries and for the designing of textiles [90]. α-amylase is used to produce modified starches for the paper industry to remove starch in the manufacturing of textiles (desising) [88]. In addition, amylases have potential industrial applications such as antisaling agent, production of cyclodextrins, sizing of textile fibres, and clarification of haziness in beer and fruit juices [4]. Variants of *B. licheniformis* α-amylase exhibit applications in textile desizing [91], as the alkaline α-amylase produed by *Bacillus* sp. A3-15 [63].

With the advent of various novel strategies in the pharmaceutical and chemical industries, amylases have been emerged as a major player in the synthesis of optically pure drugs and agrochemicals [85]. A method of curing and preventing obesity comprises orally administrating α-amylase inhibitor thereby inhibiting α-amylase activity in saliva and pancreatic juice, and reducing digestion and absorption of starch (reduction of the calorie taken from meals). Obesity can be cured or prevented effectively while taking usual diet without giving any physical or mental pain by administering oral tablets containing amylase [92]. Owing to the starch degrading ability of bacterial α-amylase—mostly from *B. subtilis*, it is widely used in pharmaceutical industry as an active

ingredient in various digestive aid preparations. Due to the presence of α-amylase, starch in the consumed food is better digested, this increases overall digestibility of food. Such digestive aid preparations are used for treatment of patients whose digesting power is reduced due to illness. These days, many such commercial formulations of digestive aids either as syrup or as tablet are seen in the shelves of many drug vebdors with very high demand.

12. CONCLUSION

Three amylases (α, β, γ) known to date in microbial world are much useful to the industry. Bifunctional industrially significant α and γ amylase fusion proteins are already available to the industry. However, a trifunctional amylase embodying the catalytic activities of all known amylases is yet to be made available to the industry. Using available crystal structures and of other sequence informations on mammalian and microbial amylases, the structure of α-amylase from novel strains of *Bacillus* can easily be predicted by homology modeling as long as we obtain apparently similar amino acid sequences. Moreover, occupational diseases associated with bacilliary amylase have to be addressed properly amidst the industrial values of microbial amylases. Occupational asthma is a major threat in workers engaged in industries, especially clothing and detergents sector. All these aspects highlight that though we use *Bacillus* with GRAS status (unlike *B. anthracis* and *B. cereus*) for amylase production, when they come to the industrial perspective, untoward risks have been sprouted up. These problems can be better solved by engineering such proteins with a view to improved enzymatic performance, including increased themostability and reduced calcium dependence. Industry demands an ideal α-amylase with increased stability, durability, reusability—especially when used at immobilised state, or a trifunctional amylase embodying the functions of all amylases with maximum activity. Such a versatile fusion protein should be useful in starch processing, starch liquefaction, fermentation, starch saccharification, cleaning, laundrying, textile desizing, baking, and biofilm removal with least expense.

13. ACKNOWLEDGEMENTS

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