

α -Amylase Production and Applications: A Review

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Abstract Amylases are important hydrolase enzymes which have been widely used since many decades. These enzymes randomly cleave internal glycosidic linkages in starch molecules to hydrolyze them and yield dextrans and oligosaccharides. Among amylases α -Amylase is in maximum demand due to its wide range of applications in the industrial front. With consumers growing increasingly aware of environmental issues, industries find enzymes as a good alternative over other chemical catalysts. α -Amylase can be produced by plant or microbial sources. Due to the advantages that microbial production offers, α -Amylase from microorganisms has been focused upon and preferred to other sources for production. The ubiquitous nature, ease of production and broad spectrum of applications make α -Amylase an industrially important enzyme. This report focuses on the microbial production of α -Amylase and its applications.

Keywords: enzymes, α -amylase, microbial production, applications

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1. Introduction

Enzymes are biological catalysts which are an indispensable component of biological reactions. The use of chemical catalysts has been followed for a very long time. Chemical catalysis though widely used was very cumbersome. The disadvantages that this method poses include need for high temperature and pressure for catalysis and the moderate specificity. These limitations were overcome by the use of enzymes. Enzymes work at milder conditions when compared to that required by chemical catalysts for operation. Also enzymes are highly specific and catalyze reactions faster than chemical catalysts [1]. Enzymes are now being used in various sectors of industry. They are used in detergents, paper industry, textile industry, food industry and many others industrial applications. Enzymes have been in use since ancient times [2] and they have been used in saccharification of starch, production of beverages like beer, treatment of digestive disorders and production of cheese from milk [3]. Among the many enzymes that are widely used α -Amylase has been in increasing demand due to its crucial role of starch hydrolysis and the applications of this hydrolytic action. The following sections elaborate on the types of amylases and their roles in enzymatic reactions.

2. Types of Amylase

2.1. α -Amylase

α -Amylase (E.C.3.2.1.1) is a hydrolase enzyme that catalyses the hydrolysis of internal α -1, 4-glycosidic

linkages in starch to yield products like glucose and maltose. It is a calcium metalloenzyme i.e. it depends on the presence of a metal co factor for its activity [4]. There are 2 types of hydrolases: endo-hydrolase and exo-hydrolase. Endo- hydrolases act on the interior of the substrate molecule, whereas exo-hydrolases act on the terminal non reducing ends [2]. Hence, terminal glucose residues and α -1, 6-linkages cannot be cleaved by α -amylase. The substrate that α -amylase acts upon is starch. Starch is a polysaccharide composed of two types of polymers – amylose and amylopectin. Amylose constitutes 20-25% of the starch molecule. It is a linear chain consisting of repetitive glucose units linked by α -1, 4-glycosidic linkage. Amylopectin constitutes 75-80% of starch and is characterized by branched chains of glucose units. The linear successive glucose units are linked by α -1, 4-glycosidic linkage while branching occurs every 15-45 glucose units where α -1, 6 glycosidic bonds are present. The hydrolysate composition obtained after hydrolysis of starch is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme. The optimum pH for activity is found to be 7.0 [4]. α -Amylase has become an enzyme of crucial importance due to its starch hydrolysis activity and the activities that can be carried out owing to the hydrolysis. One such activity is the production of glucose and fructose syrup from starch. α -Amylase catalyses the first step in this process. Previously, starch was hydrolyzed into glucose by acid hydrolysis. But this method has drawbacks like the operating conditions are of highly acidic nature and high temperatures. These limitations are overcome by enzyme hydrolysis of starch to yield high fructose syrup.

The use of enzymes in detergents formulations has also increased dramatically with growing awareness about

environment protection. Enzymes are environmentally safe and enhance the detergents ability to remove tough stains. They are biodegradable and work at milder conditions than chemical catalysts and hence preferred to the latter. There are many such applications of the enzyme which is the driving force behind the research to produce this enzyme in an optimum, safe and convenient manner [2].



Figure 1. Structure of α -Amylase [5]

2.2. β – Amylase

β -Amylase (EC 3.2.1.2) is an exo-hydrolase enzyme that acts from the nonreducing end of a polysaccharide chain by hydrolysis of α -1, 4-glucan linkages to yield successive maltose units. Since it is unable to cleave branched linkages in branched polysaccharides such as glycogen or amylopectin, the hydrolysis is incomplete and dextrin units remain. Primary sources of β -Amylase are the seeds of higher plants and sweet potatoes. During ripening of fruits, β -Amylase breaks down starch into maltose resulting in the sweetness of ripened fruit. The optimal pH of the enzyme ranges from 4.0 to 5.5. β -Amylase can be used for different applications on the research as well as industrial front. It can be used for structural studies of starch and glycogen molecules produced by various methods. In the industry it is used for fermentation in brewing and distilling industry. Also, it is used to produce high maltose syrups [6].

2.3. γ – Amylase

γ -Amylase (EC 3.2.1.3) cleaves α (1-6)glycosidic linkages, in addition to cleaving the last α (1-4)glycosidic linkages at the nonreducing end of amylose and amylopectin, unlike the other forms of amylase, yielding glucose. γ - amylase is most efficient in acidic environments and has an optimum pH of 3 [6].

3. Production of α -Amylase

3.1. Sources

α -Amylase can be isolated from plants, animals or microorganisms. The enzyme has been isolated from barley and rice plants [7]. It has been found that cassava

mash waste water is a source of α -Amylase which is active in wide range of pH and temperature [8]. In the recent past, there has been extensive research on microbial production of α -Amylase. There are 2 major reasons for the increasing interest in microbial sources:

1) The growth of microorganisms is rapid and this will in turn speed up the production of enzyme. Microorganisms are easy to handle when compared to animals and plants. They require lesser space and serve as more cost effective sources.

2) Microorganisms can be easily manipulated using genetic engineering or other means. They can be subjected to strain improvement, mutations and other such changes by which the production of α -Amylase can be optimized. Also, the microorganisms can be tailored to cater to the needs of growing industries and to obtain enzymes with desired characteristics like thermostability for example. Thermostable α -Amylases are desired as they minimize contamination risk and reduce reaction time, thus saving considerable amount of energy. Also when hydrolysis is carried out at higher temperatures, the polymerization of D-glucose to iso-maltose is minimized [9].

α -Amylase is produced by several bacteria, fungi and genetically modified species of microbes. The most widely used source among the bacterial species is the *Bacillus spp.* *B. amyloliquefaciens* and *B. licheniformis* are widely used for commercial production of the enzyme. Other species which have been explored for production of the enzyme include *B.cereus* and *B. subtilis* to name a few. α -Amylases produced from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* show promising potential in a number of industrial applications in processes such as food, fermentation, textiles and paper industries [9,10]. *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α -Amylase. Thermostability is an important characteristic as enzymatic liquefaction and saccharification of starch are performed at high temperatures (100–110°C). Thermostable amylolytic enzymes are being investigated to improve industrial processes of starch degradation and are useful for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins. Use of enzyme produced by thermophiles has the added advantage of reduced risk of contamination by mesophiles. Enzymes produced by some halophilic microorganisms are stable at high salinities and therefore could be used in many harsh industrial processes where the concentrated salt solutions are used [4]. The halophilic nature the enzyme prevents inhibition of its activity under these conditions which would otherwise occur if a normal enzyme is used. In addition, most halobacterial enzymes are considerably tolerant to high temperatures and remain stable at room temperature over long periods [4]. Halophilic amylases from halophilic bacteria such as *Chromohalobacter sp.*, *Halobacillus sp.*, *Haloarcula hispanica*, *Halomonas meridiana* [11], and *Bacillus dipsosauri* have been characterized.

Fungal sources of α -Amylase are confined to terrestrial isolates, mostly to *Aspergillus* species and to only few species of *Penicillium*, *P. brunneum* being one of them [12]. *Penicillium fellutanum* has been used in the recent past to produce α -Amylase by submerged fermentation

[12]. *Penicillium expansum* MT-1 has been used to produce the enzyme by solid state fermentation. Here, Loquat (*Eriobotrya japonica* Lindley) kernels were used as the substrate for growth of the fungi [13]. *Penicillium chrysogenum* was used as the microbial source for producing amylase by solid state fermentation using various substrates such as, corncob leaf, rye straw, wheat straw and wheat bran [14]. The fungal source used predominantly for commercial production of α -Amylase are the strains of *Aspergillus spp.* *Aspergillus oryzae*, *A. niger* and *A. awamori* are most commonly used species for commercial production among several others [9]. *Aspergillus fumigatus* has been employed for production of the enzyme by submerged fermentation technique [15].

Genetically modified organisms are also being used for production of α -Amylase. There are various methods by which microorganisms can be manipulated at a genetic level in order to improve and optimize the production of this enzyme. The microbes can be mutated by chemical agents. Nitrous acids or ethyl methane sulphonate (EMS) have been used for genetic manipulation of bacterial strains [16]. *Bacillus amyloliquefaciens* UNG-16 was subjected to mutation by both the chemical (EMS) and radiation method. The mutant strain exhibited an activity of 102.78 ± 2.22 U/ml/min which was 1.4 times greater than the parent strain [17]. α -Amylase production in *B. subtilis* Marburg was improvised by treatment with N-methyl-N'-nitro-N-nitrosoguanidine. One of the mutants, YN9, showed a threefold increase in production of the enzyme when compared to the parent strain [18]. The microbial sources and methods used to produce α -Amylase are listed in Table 1.

3.2. Production Methods

There are mainly two methods which are used for production of α -Amylase on a commercial scale. These are: 1) Submerged fermentation and 2) Solid State fermentation. The latter is a fairly new method while the former is a traditional method of enzyme production from microbes which has been in use for a longer period of time.

Submerged fermentation (SmF) employs free flowing liquid substrates, such as molasses and broths. The products yielded in fermentation are secreted into the fermentation broth. The substrates are utilized quite rapidly; hence the substrates need to be constantly replenished. This fermentation technique is suitable for microorganisms such as bacteria that require high moisture content for their growth. SmF is primarily used for the extraction of secondary metabolites that need to be used in liquid form [19]. This method has several advantages. SmF allows the utilization of genetically modified organisms to a greater extent than SSF. The sterilization of the medium and purification process of the end products can be done easily. Also the control of process parameters like temperature, pH, aeration, oxygen transfer and moisture can be done conveniently [20].

Solid state fermentation is a method used for microbes which require less moisture content for their growth.

The solid substrates commonly used in this method are, bran, bagasse, and paper pulp. The main advantage is that nutrient-rich waste materials can be easily recycled and used as substrates in this method. Unlike SmF, in this fermentation technique, the substrates are utilized very

slowly and steadily. Hence the same substrate can be used for a longer duration, thereby eliminating the need to constantly supply substrate to the process [20]. Other advantages that SSF offers over SmF are simpler equipments, higher volumetric productivity, higher concentration of products and lesser effluent generation [19]. For several such reasons SSF is considered as a promising method for commercial production of enzymes.

Table 1. Microbial sources and the mode of fermentation used for production

Bacterial source	Method	Reference
<i>B.amyloliquefaciens</i>	SSF	[7]
<i>Bacillus licheniformis</i>	SSF	[22]
<i>Bacillus coagulans</i>	SSF	[23]
<i>Chromohalobacter sp TVSP-101</i>		[24]
<i>B. polymyxa</i>	SSF	[23]
<i>B. mesentericus</i>	SSF	[23]
<i>B. vulgaris</i>	SSF	[23]
<i>B. megaterium</i>	SSF	[23]
<i>Bacillus licheniformis GCB-U8</i>	SmF	[25]
<i>Bacillus sp. PS-7</i>	SSF	[26]
<i>Bacillus licheniformis M27</i>	SSF	[27]
<i>Halobacillus sp MA-2</i>	SmF	[28]
<i>Halomonas meridiana</i>	SmF	[11]
<i>Rhodothermus marinus</i>	SmF	[29]
<i>Bacillus cereus MTCC 1305</i>	SSF	[30]
Fungal source	Method	Reference
<i>Aspergillus oryzae</i>	SSF	[31]
<i>Penicillium fellutanum</i>	SmF	[12]
<i>Thermomyces lanuginosus</i>	SSF	[32]
<i>Aspergillus niger</i>	SSF, Smf	[33]
<i>Penicillium roquefortii</i>	SSF	[6]
<i>Streptomyces rimosus</i>	SSF, Smf	[34]
<i>Aspergillus kawachii</i>	SSF, Smf	[35]
<i>Penicillium chrysogenumm</i>	SSF	[36]
<i>Penicillium janthinellum (NCIM 4960)</i>	SSF	[37]
<i>Aspergillus awamori</i>	SmF	[38]
<i>Pycnoporus sanguineus</i>	SSF	[39]

*SSF-Solid state Fermentation

* SmF- Submerged Fermentation

Fungal sources have been investigated for α -Amylase production through submerged and solid state fermentation. However, studies reveal that SSF is the most appropriate process in developing countries due to the advantages it offers which make it a cost effective production process [20]. Also SSF provides a medium that resembles the natural habitat of fungal species, unlike Smf which is considered a violation of their habitat [40].

3.3. Process Parameters

The optimum process control parameters vary depending on the microbial source, desired end product, method of fermentation employed and many other such factors.

3.3.1. Temperature

There are two temperatures that need to be in optimum range during production. They are temperature for the growth of the microbial source and optimum temperature at which maximum production of enzyme takes place. The optimum temperatures for both were studied in strains of *B. licheniformis* and *B. subtilis* 29. The optimum temperatures for growth and α -Amylase production were found to be 45°C to 46°C and 50°C , respectively. In *B. subtilis* 29 the optimum temperature for growth (42°C) was higher by 5°C than that of amylase formation (37°C) [40]. Thermophilic archaeal α -Amylases are active and

grow at high temperatures. Studies by B. Jensen, et al. (1992) and L. Bunni, et al. (1989) reveal that optimal production of amylase is between 50–55°C for the thermophilic fungal cultures such as *Talaromyces emersonii*, *Thermomonospora fusca* and *Thermomyces lanuginosus*. *Thermococcus* α -Amylases are optimally active at temperatures close to 80°C. *Pyrococcus* enzymes are optimally active around 100°C [32]. An optimum range of 95-100°C was observed to yield maximum enzyme activity in a study involving *Pyrococcus furiosus* [41]. *Penicillium fellutanum* showed an optimum activity of 98±4.6 U/ml at a temperature of 30°C [42]. An optimal temperature of 50°C was observed when temperature was varied within a range of 30-90°C for production of α -Amylase by *Aspergillus oryzae* [43]. When solid state fermentation was carried out for production of the enzyme by *Penicillium janthinellum* in a range of 30-55°C, the optimum incubation temperature was found to be 45°C [37]. The enzyme production increases with increase in temperature till it reaches the optimum. With further increase in temperature the enzyme production decreases. This may be due to the loss of moisture in the substrate which adversely affects the metabolic activities of the microbes leading to reduced growth and decline in enzyme production [37]. The optimum temperature for production of the enzyme by *Bacillus* sp. isolated from dhal industry waste was found to be 60°C [44]. The α -Amylase produced by *Clostridium acetobutylicum* was incubated at temperatures ranging from 30-60°C. The optimal activity was found to be at 45°C [45]. *Rhodothermus marinus*, a thermophilic marine microorganism exhibited optimal yield of thermostable α -Amylase at 61°C [29]. Maximum α -Amylase production of α -amylase by *Alteromonas haloplanktis* was found at an optimal temperature of 4°C [46].

3.3.2. pH

Optimum pH is a critical factor for the stability of enzyme produced. Enzymes are pH sensitive and hence care must be taken to control the pH of the production process. *Pyrococcus furiosus* produces α -Amylase which shows activity at an optimum pH of 6.5–7.5 [41]. The production of extracellular amylase by the thermophilic fungus *Thermomyces lanuginosus* was investigated in solid state fermentation (SSF). The maximum enzyme activity was 534 U/g when wheat bran was used as the substrate with optimum pH conditions of 6.0 [21]. *Bacillus amyloliquefaciens* produces the enzyme with an optimum pH of 7.0 [42]. *Halomonas meridiana* was studied for optimization of α -Amylase production. The study revealed that the amylase exhibited maximal activity at pH 7.0, being relatively stable in alkaline conditions [11]. The optimum pH for production of the enzyme by *Bacillus* sp. isolated from dhal industry waste was found to be 6.5 [44]. *Aspergillus* sp. such as *A. oryzae*, *A. ficuum* and *A. niger* exhibit optimal production at pH 5.0–6.0 in SmF [47,48,49]. *Saccharomyces cerevisiae* and *S. kluyveri* have shown maximum yield of enzyme at pH 5.0 [50,51] and pH of 7.0 respectively [52,53,54]. An initial pH range 7.5 to 8 was found to be optimal for enzyme production by *Rhodothermus marinus* [29]. Thermophilic microorganisms such as *P. woesei* and *Thermococcus profundus* exhibited optimal production of α -Amylase at pH value of 5.0 [55]. *Clostridium thermosulfurogenes*

yielded maximum enzyme at a pH of 7.0 [56]. An optimum pH of 9.0 was observed to yield enzyme with specific activity of 138 U/mg in the fungi *Preussia minima* [57]. During production of α -Amylase from *P. fellutanum* the optimum pH was found to fall within the range of 6.0 to 7.0 [12].

3.3.3. Duration of fermentation

This is a crucial factor in fermentation process. If the process is carried out for a time period shorter than the optimum duration the maximum yield cannot be obtained. The enzyme activity increases with increase in incubation time till it reaches the optimum duration. In most cases, the production of enzyme begins to decline if the incubation time is further increased. This could be due to the depletion of nutrients in the medium [31,58] or release of toxic substances. *Bacillus subtilis*, a well known producer of alpha amylase was studied for comparison between different fermentation hours and the study revealed a high yield of alpha amylase after 48 hours of fermentation [59]. Different incubation time durations were compared for yield of amylase from *Penicillium fellutanum* isolated from mangrove rhizosphere soil. The culture when incubated at 96 h, showed the maximum activity of 136 U/ ml. This was a 2 fold increase in activity when compared to 24 h incubation (68±2.3 U/ml) [12]. In an attempt to produce α -Amylase from *A. oryzae* using coconut oil cake as a substrate, the maximum activity was found to be 1752 U/gds which resulted after 72 h of incubation [43]. An optimum α -Amylase activity of 62 470 U/g was found after fermentation for 72 hours when produced by *Bacillus amyloliquefaciens* [60].

3.3.4. Substrate

3.3.4.1. Carbon Source:

Common carbon sources used as substrates include maltose, sucrose and glucose. *A. tamarii* actively synthesizes α -Amylase when cultured on maltose, starch and glycogen under static conditions. This yield was a four fold increase when compared to shaking cultures [61]. In the recent past various substrates have been investigated for use in SSF technique. The aim is to use substrates that are waste or by products of other processes in order to make the process of enzyme production environment friendly. One such substrate is oil cake. Oil cakes are byproducts of oil extraction. The chemical composition of oil cake depends upon the extraction methods used [43]. Due to the advantages that SSF offers bacterial species have also been used to produce the enzyme under SSF conditions. *Bacillus subtilis* was studied for production of α -Amylase using solid state fermentation for 48 hours at 37°C with wheat bran as substrate. The specific activity of amylase was found to be 13.14 μ mol/mg/min at 40°C [59]. Wheat bran has been used as substrate for α -Amylase production by *B. licheniformis* and *A. niger*. Studies on screening various combinations of substrates for optimum yield of enzyme by *B. amyloliquefaciens* have shown that wheat bran and groundnut oil cake in a ratio of 1:1 yield enzyme with activity of 1 671 U/g [60]. Banana waste has been used for production by *B. subtilis* [40]. Investigation of production of α -Amylase by *Bacillus cereus* MTCC 1305 revealed that among different carbon sources that were studied, glucose (0.04 g/g) showed enhanced enzyme

production (122±5) U/g). On the other hand supplementation of different nitrogen sources (0.02 g/g) showed decline in enzyme production [30].

3.3.4.2. Nitrogen Source

The nitrogen source used for production of α -Amylase may be organic or inorganic. Few of the inorganic nitrogen sources include ammonium sulphate, ammonium chloride and ammonium hydrogen phosphate. Most commonly used organic sources of nitrogen include peptone, yeast extract and soyabean meal [44]. In a study of production of the enzyme by *Aspergillus oryzae*, NaNO₃ (0.9%) and malt (1%) gave highest yield of enzyme among many other nitrogen sources [62]. In another study of *Aspergillus oryzae* organic sources did not yield good results. Inorganic sources such as ammonium chloride, ammonium phosphate, ammonium sulphate resulted in low yield of enzyme. Only when the inorganic sources were replaced by ammonium nitrate and sodium nitrate did the yield increase marginally [31]. In a study conducted on *Penicillium fellutanum* the maximum activity of 150 U/ml was obtained when peptone was used as the nitrogen source. This was about 9% higher than the activity obtained by using yeast, meat and casein [12]. Peptone is usually the most high yielding nitrogen source when employed in solid- state fermentation. A maximum production of 812 U/g was achieved when peptone was employed as the nitrogen source for α -amylase production by *P. expansum*. Among the inorganic nitrogen sources, ammonium salts showed better results when compared to sodium nitrate [58]. Studies reveal that peptone, sodium nitrate and casein hydrolysate are good nitrogen sources for *A. fumigatus* [63] and *A.niger* [49]. Among the nitrogen sources tested for *Thermomyces lanuginosus*, peptone showed the best results for enzyme production (414 U/g). Tryptone (356 U/g) and meat extract (338 U/g) also yielded good amount of enzyme [64].

3.3.5. Moisture

Optimum levels of initial moisture content may vary depending on the microbial source used. For fungal sources the moisture content required is less whereas bacterial sources need more moisture content for high yield of the enzyme [31]. The optimum level can be determined by determining the enzyme yield within a range of initial moisture content. In a study of production of α -Amylase by *Penicillium janthinellum* the moisture content was varied within a range of 20- 80% by varying the amount of salt solution used in moistening the substrate particles. The study revealed that with wheat bran as the substrate, the maximum yield of enzyme was of 295 U/gds with 60% of initial moisture content. The yield decreased to 220 U/gds at 80% (w/w) moisture content [37]. For *Aspergillus oryzae* the maximum yield was found to be 9000 U/gds at an initial moisture content of 60% which declined drastically when the moisture content was increased to 70% [31]. The optimal initial moisture content may also vary with substrate in addition to the microbial source used. This may be due to fact that substrates with better water holding capacity require lower moisture content for optimal production of the enzyme when compared to others. Production of α -Amylase from *Penicillium chrysogenum* was carried out using various agricultural by- products like wheat bran (WB), corncob

leaf (CL), rye straw (RS) and wheat straw (WS). Enzyme yields were 20, 34, 40 and 127 U/mL with CL, RS, WS and WB at 75, 55, 65 and 65% of initial moisture content, respectively (14). For *B. amyloliquefaciens*, studies were carried out to optimize the process parameters in which the initial moisture content was varied from 55- 90% for optimization. An optimal production of 3677 U/g of α -Amylase was found at 85% of initial moisture content using a combination of wheat bran and groundnut oil cake as the substrate [60]. The enzyme yield usually increases with increase in initial moisture content reaching an optimal level followed by decrease in enzyme yield with further increase in moisture content. At low moisture content there is high water tension and low solubility of the nutrients which causes the low yield of enzyme. With increase in moisture content the swelling of substrate takes place which ensures better uptake of nutrients by the microbes. But with increase of moisture content further the enzyme yield decreases owing to many reasons. The inter particle distances reduce and can result in agglomeration of substrate particles. Also the reduction in gas volume and gas diffusion results in impaired oxygen transfer [31,37].

4. Determination of enzyme activity

The enzyme activity is determined by measuring the reducing sugars released as a result of the action of α -Amylase on starch. Another method is to measure the extent of hydrolysis by reading the absorbance of starch-iodine complex. Few of the commonly used methods for enzyme assay are discussed below.

4.1. Dinitrosalicylic Acid Method (DNS)

In the dinitrosalicylic acid method, aliquots of the substrate stock solution are mixed with the enzyme solution. Followed by 10 min of incubation at 50°C, DNS reagent is added to the test tube and the mixture is incubated in a boiling water bath for 5 min. After cooling to room temperature, the absorbance of the supernatant at 540 nm is measured. The A540 values for the substrate and enzyme blanks are subtracted from the A540 value for the analyzed sample [45,65, 66]. In a study on alkalophilic α -Amylase from *Bacillus* strain GM8901, the enzyme assay was done by measuring the reducing sugars by DNS method and the activity was found to be a maximum of 0.75 U ml⁻¹ after incubation of 24 h [65].

4.2. Nelson – Somogyi (NS) Method

In the NS method, an aliquot of stock solution of substrate is heated at 50°C for 5min. Preheated (50°C for 5 min) enzyme solution is added to the substrate. This reaction mixture is incubated at 50°C and the reaction is carried out for 10min. After incubation Somogyi copper reagent is added to terminate the reaction. This is then incubated in boiling water bath for 40 min & cooled to room temperature. In the next step Nelson arsenomolybdate reagent is added and incubated for 10 min at room temperature. Finally water is added and the mixture is centrifuged at 13,000 rpm for 1 min and absorbance of supernatant is read at 610 nm [66]. Haloalkaliphilic α -Amylase isolated from archaebacterium

Natronococcus sp. Strain Ah-36 was subjected to NS method of enzyme assay and activity of 0.01 U/ml was recorded in the end of 35 h of incubation. The maximum activity was found to be 0.12 U/ml after 90 h and was retained even after 110 h [67].

4.3. Determination of Activity Using Iodine

The hydrolytic activity of α -Amylase can be determined based on the principle that starch and iodine react to form a blue colored complex. On hydrolysis of starch this complex changes to a reddish brown colored one. The absorbance can be read after the enzyme substrate reaction has been terminated. This gives a measure of the extent of hydrolysis of starch by α -Amylase [2].

4.3.1. Dextrinizing Activity

In a study involving characterization of thermophilic α -Amylase from *P. furiosus*, an adaptation of the assay described by Manning and Campbell (1961) was employed to carry out the enzyme assay. The crude enzyme sample is incubated at 92°C with soluble starch to carry out the reaction for 10 min. The reaction is terminated by cooling the reaction mixture in ice water. Iodine (KI, Iodine) is added to form a colored complex with the starch in the reaction mixture. This complex is diluted with water in order to bring the color to a measurable range that can be read at 600 nm [41].

4.3.2. Indian Pharmacopoeia Method

This method is employed to measure the enzyme activity in terms of the amount of starch (grams) digested by enzyme of a given volume. In this method various dilutions of enzyme solution in incubated with starch at 40°C for 1 h after forming the colored complex by addition of iodine, the tube that shows no blue color is used to calculate the enzyme activity [2].

4.4. Reduction in Viscosity of Starch Suspension

This method is used to determine the quality of flour in the baking industry. Two methods used to measure enzyme activity in terms of decreasing viscosity of starch suspension are: falling number (FN) method and Amylograph/Farinograph test. In the falling number method, the enzyme substrate preparations are assayed at 100°C. Normally malted flour has a falling number of around 400. The amylograph test employs the principle of the relationship between peak viscosity of starch slurry and the enzyme activity. The lesser the viscosity of the starch slurry, more is the activity of the enzyme. Values of 400-/600 Brabender units of the Farinograph are found optimal for flour used to bake bread [2].

5. Purification of α -Amylase

Enzymes used for industrial applications are usually crude preparations and require less downstream processing. Whereas the enzymes used clinical and pharmaceutical industry need to be highly purified. Also when used for study of structure function relationships and biochemical properties the enzymes have to be in

purified form [2]. Purification methods commonly employed are precipitation, chromatography and liquid-liquid extraction depending on the properties of the enzyme desired. A combination of the above methods is used in a series of steps to achieve high purity. The number of steps involved in purification will depend on the extent of purity that is desired [30,59,60,61]. The crude extracellular enzyme sample can be obtained from the fermented mass by filtration and centrifugation. In the case of intracellular enzymes, raw corn starch may be added followed by filtration and subsequent steps. The crude amylase enzyme can be precipitated and concentrated using ammonium sulphate precipitation or organic solvents. The precipitated sample can be subjected to dialysis against water or a buffer for further concentration [68]. This can be followed by any of the chromatographic techniques like ion exchange, gel filtration and affinity chromatography for further separation and purification of the enzyme. In a method of purification of the enzyme produced by *Aspergillus falvus* var. *columnaris*, the enzyme was precipitated was followed by dialysis and then column chromatography [68]. In a study of Amylase production by *Preussia minim*, the sample was precipitated using trichloroacetic acid (TCA)/acetone and applied to a Sephadex G-200 gel filtration column. The resulting fractions were pooled and further applied to a DEAE-Sepharose ion exchange column [57]. In a study of purification and characterization of an extracellular α -Amylase from *Clostridium perfringens* Type A, crude enzyme concentrate was prepared by precipitation with polyethylene glycol. This concentrated sample was then separated by DEAE Sephacel chromatography resulting in three distinct amylolytic peaks. Fractions from a single peak were then collected and chromatographed on the same column, followed by separation of obtained active fractions on a Sephacryl S-100 HR column [69]. Purification of α -Amylase produced by a mutant *Bacillus subtilis* strain was done in a series of steps employing precipitation with 80% ammonium sulfate, TSK Toyopeal column chromatography, ultrafiltration, dialysis and SP Sepharose column chromatography [70]. In case of thermotolerant amylases, the cell extract obtained after centrifugation which is free of cell debris can be subjected to high temperatures, in order to denature thermolabile proteins. For purification of α -Amylase produced by *Thermotoga maritima* MSB8, this step was followed by anion exchange chromatography. The purity was then analysed by using SDS-PAGE [71]. The purity can also be analysed by size exclusion chromatography wherein the molecular weight of the purified protein can be determined.

6. Characterization of α -Amylase

Once the enzyme has been purified, characterization of the enzyme is carried out. This can be done by determining the hydrolysates obtained after enzyme action on starch by PAGE. The purified enzyme sample along with molecular markers like BSA (67 kDa) and ovalbumin (43 kDa), are run on the gel. The resulting bands are then stained using staining agents like Coomassie Brilliant Blue [72] and silver nitrate [73] and visualized. Various

types of electrophoretic units are available for PAGE [74]. The electrophoretic separation also indicates the purity and homogeneity of the enzyme obtained. In a study of α -Amylase produced by *Bacillus* MNJ23 strain the molecular mass of the enzyme was characterized by SDS-PAGE (12% acrylamide gel) using Bio-Rad miniprotein II electrophoresis unit [74]. Characterization of the enzyme produced by *Clostridium acetobutylicum* ATCC 824 was carried out by SDS-PAGE in a Phast Gel gradient (10 to 15%) with the Pharmacia Phast system with standard proteins ranging from 14,400 to 94,000 in molecular weight for comparison [73]. For determination of molecular weight of purified enzyme produced by mutant *Bacillus subtilis*, SDS-PAGE (10%) was carried out [70].

The molecular mass of the enzyme can also be determined using size exclusion chromatography [75]. A study on intracellular α -Amylase produced by *Streptococcus bovis* 148 showed the appearance of a single band on a SDS-PAGE gel which confirmed the purification to homogeneity. The molecular mass after purification was found to be 57,000 Da. The molecular mass was determined as 55,000 Da from gel filtration method. This indicated that the intracellular α -Amylase exists in a monomeric form [76]. The characterization of molecular weight of α -Amylase produced by in *Pyrococcus furiosus* by G Dong, C Vieille et al (1997) was carried out using gel filtration chromatography that employed a column containing Sephacryl S200 with carbonic anhydrase (29,000), bovine serum albumin (66,000), alcohol dehydrogenase (150,000), and Blue Dextran (2,000,000) as standards [76].

Isoelectric focusing is another method used in addition to the gel based characterization technique. The isoelectric point of the enzyme can be determined by using protein markers of broad range of pI of values [73]. Characterization by this method was used for α -Amylase produced by *C. thermohydrosulfuricum* E 101-69. The crude enzyme sample was concentrated using ultrafiltration. This was followed by isoelectric focusing on a gel with pI range of 3.5-5.0. The Pharmacia low-pI-calibration-kit was used for standards [77]. For determination of

isoelectric point of α -Amylase produced by *Clostridium acetobutylicum* ATCC 824, Phast Gel IEF 3-9 with the Pharmacia Phast system with protein markers (Pharmacia) of pI 3 to 10 as standards was used [73].

7. Industrial Applications of α -Amylase

α -Amylase is gaining increased attention due to its starch hydrolyzing properties and the activities that can be carried out owing to this property. There are many potential and widely used applications of this enzyme on the industrial front. Enzymes have replaced the previously used chemical methods of hydrolysis in various industrial sectors to make the process environment friendly and make processes easier. The industrial applications of α -Amylase and the microbial sources used for its production are described in Table 2.

7.1. Production of Fructose and Glucose by Enzymatic Conversion of Starch

Starch is used in the production of fructose and glucose syrups [78]. This process involves three steps: Gelatinization, Liquefaction, and Saccharification. Gelatinization involves the dissolving of starch granules in water to form a viscous starch suspension. The amylase and amylopectin are dispersed into the water on dissolution. Liquefaction of starch is its partial hydrolysis into short chain dextrans by α -Amylase resulting in reduction of the viscosity of the starch suspension. Saccharification is the production of glucose and fructose syrup by further hydrolysis. This is carried out by glucoamylase which acts as an exo-amylase by cleaving the α -1, 4 glycosidic linkages from the non reducing terminal. The action of pullulanase along with glucoamylase yields high glucose syrup. This high glucose syrup can then be converted into high fructose syrup by isomerization catalysed by glucose isomerase. The fructose syrup obtained is used as a sweetener, especially in the beverage industry.

Table 2. Industrial applications of α -Amylase and the microbial source used

Industrial Application	Microbial source	Role of enzyme	Reference
Starch conversion	<i>B.amyloliquefaciens</i> , <i>B.licheniformis</i>	Hydrolysis of starch into dextrans forming a less viscous starch suspension	[4]
Bakery Industry	<i>B.stearothermophilus</i>	Converting starch in dough to smaller fermentable sugars	[2]
Detergent Industry	<i>B.licheniformis</i>	Digests starch containing foods to water soluble dextrin	[2]
Textile Industry	<i>Bacillus sp</i>	Used in removal of starch sizing agent from woven fabric	[4]
Fuel alcohol Production	<i>E.coli</i> , <i>B.subtilis</i>	Converting starch in to smaller fermentable sugars which are acted upon by yeast to produce alcohol	[4]

The hydrolysis of starch for this purpose was previously carried by acid hydrolysis followed by saccharification using enzymes. This method had many drawbacks. The acidic nature of the process required corrosion resistant material to be used for the equipment and the high temperatures would inactivate the thermolabile enzymes if the hot starch hydrolysate passes into subsequent steps. Hence, enzymatic hydrolysis is a preferred method. The α -Amylase used in the liquefaction step can be produced from various microbial sources. Thermostable amylases can be employed for hydrolysis at a high temperature. *Bacillus Stearothermophilus*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Pyrococcus furiosus* are few of

the many microbial sources used to produce α -Amylase that is used in starch conversion [2,4,79].

7.2. Bakery Industry

α -Amylase is added to the dough in bread baking process. This causes the starch to hydrolyze into small dextrans which can further be fermented by yeast. This increases the rate of fermentation. Also the starch hydrolysis decreases the viscosity of the dough, thus improving its texture and increasing loaf volume by rising of dough.

Once the baking is done, there may be changes during storage of baked products. All undesirable changes like increase of crumb firmness, loss of crispness of the crust, decrease in moisture content of the crumb and loss of bread flavor together are called staling. The enzyme is also used as an anti staling agent to improve the shelf life and softness retention of baked goods [2,4,79]. Though it has an anti staling effect, a slight overdose may result in gummyness of the bread. This is caused due to production of branched dextrans [80]. In such cases pullulanase is used in combination with amylase resulting in specific hydrolysis of compounds responsible for the gummy nature of amylase treated bread [79].

7.3. Detergent Industry

The use of enzymes in detergents has increased with the changing methods of dishwashing and laundry. Consumers prefer to use cold water and mild conditions which requires the detergent to work in those imitations. Earlier the chemicals used in detergents caused harm when ingested and the conditions of dishwashing were very harsh. Hence enzymes showed the industry an alternative path. The enzymes are environmentally safe and work at mild conditions. α -Amylase is used to digest the starch containing food particles into smaller water soluble oligosaccharides. Starch can attract soil particles on to the clothes. Hence removal of starch is also important to maintain the whiteness of clothes. The stability of α -Amylase at low temperature and alkaline pH contributes to its extensive use in detergents.

The disadvantage of using α -Amylase is the calcium dependency and oxidant sensitivity of the enzyme. This limitation can be overcome by using α -Amylase from genetically modified organisms. Scientists from Novozymes and Genencore International, two major suppliers of detergent enzymes, have worked on this. They replaced the oxidant sensitive amino acid residue *met* at position 197 by *leu* in *B. licheniformis* amylase which resulted in an amylase with improved resistance against oxidative compounds [2].

7.4. Desizing of Textiles

Modern production processes in the textile industry can cause breaking of the warp thread. To strengthen the thread, sizing agents are used which strengthen the thread by forming a layer on it and can be removed after the fabric is woven. Starch is a preferred sizing agent as it is easily available, cheaper and can be easily removed from the fabric. The layer of starch is subjected to hydrolysis in the desizing process where α -Amylase is employed to cleave starch particles randomly into water soluble components that can be removed by washing. The enzyme acts specifically on the starch molecules alone, leaving the fibers unaffected [2,4].

7.5. Paper Industry

Like textiles, paper is also treated with sizing agents to protect it from mechanical strain during processing. The sizing also contributes to better quality of the paper in terms of strength, smoothness, writing and erasability. Starch is commonly used as the sizing agent. The role of α -Amylase in the paper industry is the partial hydrolysis

of starch to make it less viscous in a batch or a continuous process. This is owing to the highly viscous nature of natural starch making it unsuitable for coating on paper [2,4].

7.6. Fuel Alcohol Production

Among biofuels, ethanol is most widely used. As starch is an economical starting material, it is used for the production of ethanol as a biofuel. This is done in a series of steps. Firstly, the starch is subjected to liquefaction to form a viscous starch suspension. This is followed by the saccharification process where the starch is hydrolyzed by α -Amylase to yield fermentable sugars. These sugars are then fermented by yeast to produce alcohol. As an improvisation of this process, protoplast fusion between the amylolytic yeast *Saccharomyces fibuligera* and *S. cerevisiae* was performed to obtain a new yeast strain that can directly produce the biofuel from starch, eliminating the need for a saccharification step [81].

8. Conclusion

The increasing importance of sustainable development has inspired man to use enzymes for various reactions as they are biodegradable and can be produced using biological sources. α -Amylase can be produced using microbes by SSF which employs by products and waste products of other processes. The enzyme has crucial applications including the production of fructose syrup, environmentally safe detergents, and baked products. Living in an era of depleting fossil fuels with a desperate need to produce alternate forms of energy, this enzyme is a ray of hope. It is used for biofuel production with starch as a raw material. As the production yields an industrially important enzyme and helps keep the environment clean, more researches are focusing on the microbial production of the enzyme.

References

- [1] Prasad Nooralabettu Krishna, "Enzyme Technology: Pacemaker of Biotechnology", PHI Learning Pvt. Ltd., 2011.
- [2] Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., & Chauhan, B. "Microbial α -amylases: a biotechnological perspective", *Process Biochemistry*, 38 (11), 1599-1616, (2003).
- [3] Drauz, K., Gröger, H., & May, O. (Eds.) *Enzyme catalysis in organic synthesis: a comprehensive Handbook*, John Wiley & Sons, (2012), Souza, P. M. D., "Application of microbial α -amylase in industry-A review", *Brazilian journal of microbiology*, 41 (4), 850-861, May (2010).
- [4] rcsb.org (http://rcsb.org/pdb/images/1hny_bio_r_500.jpg?bioNum=1)
- [5] Das, S., Singh, S., Sharma, V., & Soni, M. L. "biotechnological applications of industrially important amylase enzyme". *International Journal of Pharma & Bio Sciences*, 2 (1), March (2011).
- [6] Sivaramakrishnan, S., Gangadharan, D., Nampoothiri, K. M., Soccol, C. R., & Pandey, A. " α -Amylases from Microbial Sources--An Overview on Recent Developments". *Food Technology & Biotechnology*, 44 (2), March (2006).
- [7] Oboh, G. "Isolation and characterization of amylase from fermented cassava (*Manihot esculenta* Crantz) wastewater". *African journal of biotechnology*, 4 (10).October (2005).
- [8] Pandey, A., Nigam, P., Soccol, C. R. V. T., Soccol, V., Singh, D., & Mohan, R."Advances in microbial amylases". *Biotechnology and Applied Biochemistry*, 31, 135-152, (2000).

- [9] Konsoula, Z, Liakopoulou-Kyriakides M, "Co-production of alpha-amylase and beta-galactosidase by *Bacillus subtilis* in complex organic substrates." *Bioresource Technology*, 98, 150-157, (2007).
- [10] Coronado, M. J., Vargas, C., Hofemeister, J., Ventosa, A., & Nieto, J. J. "Production and biochemical characterization of an α - amylase from the moderate halophile *Halomonas meridiana*". *FEMS microbiology letters*, 183 (1), 67-71, (2000).
- [11] Kathiresan, K., & Manivannan, S. "A- Amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil". *African journal of Biotechnology*, 5 (10), (2006).
- [12] Erdal, S. E. R. K. A. N., & Taskin, M. E. S. U. T. "Production of alpha-amylase by *Penicillium expansum* MT-1 in solid-state fermentation using waste Loquat (*Eriobotrya japonica* Lindley) kernels as substrate". *Romanian Biotechnological Letters*, 15 (3), 5342-5350, (2010).
- [13] Balkan, B., & Ertan, F. "Production of a-amylase from *Penicillium chrysogenum* under solid-state fermentation by using some agricultural by-products". *Food Technol. Biotechnol.*, 45, 439-442, (2007).
- [14] Goto, C.E.; Barbosa, E.P.; Kistner, L.C.; Moreira, F.G.; Lenartovicz, V.;Peralta, R.M. "Production of amylase by *Aspergillus fumigatus* utilizing alpha-methyl-D-glycoside, a synthetic analogue of maltose, as substrate". *FEMS Microbiol Lett* 167, 139-143, (1998).
- [15] Bin, G., X. Laisu, D. Youfang and L. Yanquan, "Screening of alpha amylase high-producing strains from *Bacillus subtilis*", *Journal of Zhejiang*, 23, 88-92, (1999).
- [16] Haq, I., Ali, S., Javed, M. M., Hameed, U., Saleem, A., Adnan, F., & Qadeer, M. A. "roduction of alpha amylase from a randomly induced mutant strain of *Bacillus amyloliquefaciens* and its application as a desizer in textile industry" *Pak J Bot.* 42 (1), 473-484, (2010).
- [17] Y. Yoneda and B. Maruo, "Mutation of *Bacillus subtilis* Causing Hyperproduction of α -Amylase and Protease, and Its Synergistic Effect", *Journal of Bacteriology*, 124, 48-54, (1975)
- [18] Pandey, A., Selvakumar, P., Soccol, C. R., & Nigam, P. "Solid state fermentation for the production of industrial enzymes" *Current science*, 77 (1), 149-162, (1999).
- [19] Couto, S. R., & Sanromán, M. A. "Application of solid-state fermentation to food industry-a review" *Journal of Food Engineering*, 76 (3), 291-302, (2006).
- [20] Kunamneni, A., Permaul, K., & Singh, S. "Amylase production in solid state fermentation by the thermophilic fungus *Thermomyces lanuginosus*". *Journal of bioscience and bioengineering*, 100(2), 168-171, (2005).
- [21] Bhargav, S., Panda, B. P., Ali, M., & Javed, S. "Solid-state fermentation: an overview". *Chemical and Biochemical Engineering Quarterly*, 22 (1), 49-70, (2008).
- [22] Babu, K. R., & Satyanarayana, T. "A-Amylase production by thermophilic *Bacillus coagulans* in solid state fermentation". *Process Biochemistry*, 30 (4), 305-309, (1995).
- [23] B. Prakash, M. Vidyasagar, M.S. Madhukumar, G. Muralikrishna, K. Sreeramulu, "Production, purification, and characterization of two extremely halotolerant, thermostable, and alkali-stable a-amylases from *Chromohalobacter sp.* TVSP 101", *Process Biochemistry*, 44, 210-215, (2009)
- [24] Ashraf, H., Iqbal, J., & Qadeer, M. A. "Production of alpha amylase by *Bacillus licheniformis* using an economical medium". *Bioresource Technology*, 87 (1), 57-61, (2003).
- [25] Sodhi, H.K.; Sharma, K.; Gupta, J.K.; Soni, S.K. "Production of a thermostable α -amylase from *Bacillus sp.* PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production". *Process Biochemistry*, 40, 525-534, (2005).
- [26] Ramesh, M. V., & Lonsane, B. K. "Critical importance of moisture content of the medium in alpha-amylase production by *Bacillus licheniformis* M27 in a solid-state fermentation system". *Applied microbiology and biotechnology*, 33 (5), 501-505, (1990).
- [27] Amoozegar, M. A., Malekzadeh, F., & Malik, K. A. "Production of amylase by newly isolated moderate halophile, *Halobacillus sp.* strain MA-2". *Journal of microbiological methods*, 52 (3), 353-359, (2003).
- [28] Gomes, I., Gomes, J., & Steiner, W. "Highly thermostable amylase and pullulanase of the extreme thermophilic eubacterium *Rhodothermus marinus*: production and partial characterization". *Bioresource technology*, 90 (2), 207-214, (2003).
- [29] Anto, H., Trivedi, U., & Patel, K. "A-Amylase production by *Bacillus cereus* MTCC 1305 using solid-state fermentation". *Food Technology and Biotechnology*, 44 (2), 241-245, (2006).
- [30] Sivaramakrishnan, S., Gangadharan, D., Nampoothiri, K. M., Soccol, C. R., & Pandey, A. "Alpha amylase production by *Aspergillus oryzae* employing solid-state fermentation". *Journal of scientific and industrial research*, 66 (8), 621, (2007).
- [31] Lévêque, E., Janeček, Š., Haye, B., & Belarbi, A. "Thermophilic archaeal amylolytic enzymes". *Enzyme and Microbial Technology*, 26 (1), 3-14, (2000).
- [32] Uggade, A., Nandeshwar, A., and Samant, L., "Assessment of fungal protease enzyme from French bean using *A. niger* by Solid State Fermentation", *Journal of Microbiology and Biotechnology Research*, 1, 45-51, (2011).
- [33] Yang, S. S., & Wang, J. Y. "Protease and amylase production of *Streptomyces rimosus* in submerged and solid state cultivations". *Botanical Bulletin of Academia Sinica*, 40, 259-265, (1999).
- [34] Sudo, S., Ishikawa, T., Sato, K., & Oba, T. "Comparison of acid-stable α -amylase production by *Aspergillus kawachii* in solid-state and submerged cultures". *Journal of fermentation and bioengineering*, 77 (5), 483-489, (1994).
- [35] Balkan, B., & Ertan, F. "Production of a-amylase from *Penicillium chrysogenum* under solid-state fermentation by using some agricultural by-products". *Food Technol. Biotechnol.*, 45, 439-442, (2007).
- [36] Sindhu, R., Suprabha, G. N., & Shashidhar, S. "Optimization of process parameters for the production of a-amylase from *Penicillium janthinellum* (NCIM 4960) under solid state fermentation". *African Journal of Microbiology Research*, 3 (9), 498-503, September (2009).
- [37] Prakasham, R. S., Subba Rao, C., Sreenivas Rao, R., & Sarma, P. N. "Enhancement of acid amylase production by an isolated *Aspergillus awamori*". *Journal of applied microbiology*, 102 (1), 204-211, (2007).
- [38] De Almeida Siqueira, E. M., Mizuta, K., & Giglio, J. R. "*Pycnoporus sanguineus*: a novel source of α -amylase". *Mycological research*, 101 (2), 188-190, (1997).
- [39] Saito, N. A. R. I. M. A. S. A., & Yamamoto, K. A. T. S. U. J. I. "Regulatory factors affecting alpha-amylase production in bacillus licheniformis". *Journal of bacteriology*, 121 (3), 848-856, (1975).
- [40] Kenneth A. Laderman, Bradley R. Davis, Henry C. Krutzsch, Marc S. Lewis, Y. V. Griko, Peter L. Privalov, and Christia B. Anfinsen, "The Purification and Characterization of an Extremely Thermostable α -Amylase from the Hyperthermophilic Archaeobacterium *Pyrococcus furiosus*", *Journal of Biological Chemistry*, 268 (32), 24394-24401, (1993).
- [41] Haki, G. D., & Rakshit, S. K. "Developments in industrially important thermostable enzymes: a review". *Bioresource Technology*, 89 (1), 17-34, (2003).
- [42] Ramachandran, S., Patel, A. K., Nampoothiri, K. M., Chandran, S., Szakacs, G., Soccol, C. R., & Pandey, A. "Alpha amylase from a fungal culture grown on oil cakes and its properties". *Brazilian Archives of Biology and Technology*, 47 (2), 309-317, June 2004.
- [43] Thippeswamy, S., Girigowda, K., & Mulimani, V. H. "Isolation and identification of alpha-amylase producing *Bacillus sp.* from dhal industry waste". *Indian Journal of Biochemistry and Biophysics*, 43 (5), 295, (2006).
- [44] Paquet, V. E. R. O. N. I. Q. U. E., Croux, C. H. R. I. S. T. I. A. N., Goma, G. E. R. A. R. D., & Soucaille, P. H. I. L. I. P. P. E. "Purification and characterization of the extracellular alpha-amylase from *Clostridium acetobutylicum* ATCC 824". *Applied and Environmental Microbiology*, 57 (1), 212-218, (1991).
- [45] Feller, G., Le Bussy, O., & Gerday, C. Expression of psychrophilic genes in mesophilic hosts: assessment of the folding state of a recombinant α -amylase. *Applied and Environmental Microbiology*, 64 (3), 1163-1165, (1998).
- [46] S. Hayashida, Y. Teramoto, "Production and characteristics of raw-starch-digesting-a-amylase from a protease negative *Aspergillus ficuum* mutant", *Applied and environmental microbiology*, 52, 1068-1073, (1986).
- [47] M. Carlsen, J. Nielsen, J. Villadsen, "Growth and a-amylase production by *Aspergillus oryzae* during continuous cultivations", *Journal of Biotechnology*, 45, 81-93, (1996).
- [48] S. Djekrif-Dakhmouche, Z. Gheribi-Aoulmi, Z. Meraihi, L. Bennamoun, "Application of a statistical design to the optimization of culture medium for a-amylase production by *Aspergillus niger* ATCC 16404 grown on orange waste powder", *Journal of Food Engineering*, 73, 190-197, (2005).

- [49] K. Moller, M.Z. Sharif, L. Olsson, "Production of fungal α -amylase by *Saccharomyces kluyveri* in glucose-limited cultivations", *Journal of Biotechnology*, *111*, 311-318, (2004).
- [50] Knox, A. M., du Preez, J. C., & Kilian, S. G. "Starch fermentation characteristics of *Saccharomyces cerevisiae* strains transformed with amylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*". *Enzyme and Microbial Technology*, *34* (5), 453-460, (2004).
- [51] M.S. Tanyildizi, D. Ozer, M. Elibol, "Optimization of α -amylase production by *Bacillus* sp. using response surface methodology", *Process Biochemistry*, *40*, 2291-2296, (2005).
- [52] Syu, M. J., & Chen, Y. H. "A study on the α -amylase fermentation performed by *Bacillus amyloliquefaciens*". *Chemical Engineering Journal*, *65* (3), 237-247, (1997).
- [53] Ashraf, H., MA, Q., & Iqbal, J. "Pearl millet, a source of alpha amylase production by *Bacillus licheniformis*". *Bioresource technology*, *96* (10), 1201-1204, (2005).
- [54] K. Vieille, G.J. Zeikus, "Hyperthermophilic enzymes: Sources, uses, and molecular mechanisms of thermostability", *Microbiology and Molecular Biology Review*, *65*, 1-43, (2001).
- [55] M.V. Swamy, G. Seenayya. "Thermostable pullulanase and α -amylase activity from *Clostridium thermosulfurogenes* SV9 optimization of culture conditions for enzyme production" *Process Biochemistry*, *31*, 157-162, (1996).
- [56] Zaferanloo, B., Bhattacharjee, S., Ghorbani, M. M., Mahon, P. J., & Palombo, E. A. "Amylase production by *preussia minima*, a fungus of endophytic origin: optimization of fermentation conditions and analysis of fungal secretome by LC-MS". *BMC microbiology*, *14* (1), 55, (2014).
- [57] Erdal, S. E. R. K. A. N., & Taskin, M. E. S. U. T. "Production of alpha-amylase by *Penicillium expansum* MT-1 in solid-state fermentation using waste Loquat (*Eriobotrya japonica* Lindley) kernels as substrate". *Romanian Biotechnological Letters*, *15* (3), 5342-5350, (2010).
- [58] Raul, D., Biswas, T., Mukhopadhyay, S., Kumar Das, S., & Gupta, S. "Production and Partial Purification of Alpha Amylase from *Bacillus subtilis* (MTCC 121) Using Solid State Fermentation. *Biochemistry Research International*, *2014*. (2014).
- [59] Gangadharan, D, Sivaramakrishnan, S, Nampoothiri K M and Pandey A: "Solid Culturing of *Bacillus amyloliquefaciens* for Alpha Amylase Production.", *Food Technology & Biotechnology*, *44*, 269-274, Apr-Jun2006.
- [60] Moreira F G, de Lima F A, Fazzano Pedrinho S R, Lenartovicz V, Giatti C, de Souza M, Peralta R M, "Production of amylases by *Aspergillus tamarit*", *Revista de Microbiologia*, *30*, 157-162, (1999)
- [61] Kundu A. K. and Das S., "Production of Amylase in Liquid Culture by a Strain of *Aspergillus oryzae*", *Applied Microbiology*, *19*, No. 4, 598-603, April (1970)
- [62] Goto, C.E.; Barbosa, E.P.; Kistner, L.C.; Moreira, F.G.; Lenartovicz, V.;Peralta, R.M. "Production of amylase by *Aspergillus fumigatus* utilizing alpha-methyl-D-glycoside, a synthetic analogue of maltose, as substrate". *FEMS Microbiol Lett* *167*, 139-143, (1998).
- [63] Kunamneni, A., Permaul, K., & Singh, S. "Amylase production in solid state fermentation by the thermophilic fungus *Thermomyces lanuginosus*". *Journal of bioscience and bioengineering*, *100* (2), 168-171, (2005).
- [64] Kim, T. U., Gu, B. G., Jeong, J. Y., Byun, S. M., & Shin, Y. C. "Purification and Characterization of a Maltotetraose-Forming Alkaline (alpha)-Amylase from an Alkalophilic *Bacillus* Strain, GM8901". *Applied and environmental microbiology*, *61* (8), 3105-3112, (1995).
- [65] Gusakov, A. V., Kondratyeva, E. G., & Sinitsyn, A. P. "Comparison of two methods for assaying reducing sugars in the determination of carbohydrase activities". *International journal of analytical chemistry*, *2011*, (2011).
- [66] Kobayashi T, Kanai H, Hayashi T, Akiba T, Akaboshi R and Horikoshi K, "Haloalkaliphilic Maltotriose-Forming α -Amylase from the Archaeobacterium *Natronococcus* sp. Strain Ah-36", *Journal of Bacteriology*, *174*, 3439-3444, (1992).
- [67] El-Safey, E. M., & Ammar, M. S. "Purification and Characterization of α -amylase Isolated from *Aspergillus falvus* var. *Columnaris*". *Ass. Univ. Bull. Environ. Res*, *7* (1), 93-100, (2004).
- [68] Shih N-J and Labbe R G. , "Purification and Characterization of an Extracellular α -Amylase from *Clostridium perfringens* Type A", *Applied and Environmental Microbiology*, *61*, 1776-1779, (1995).
- [69] Demirkan, E. "Production, purification, and characterization of α -amylase by *Bacillus subtilis* and its mutant derivatives". *Turk J Biol*, *35*, 705-712, (2011).
- [70] Ballschmiter, M., Fütterer, O., & Liebl, W. "Identification and characterization of a novel intracellular alkaline α -amylase from the hyperthermophilic bacterium *Thermotoga maritima* MSB8". *Applied and environmental microbiology*, *72* (3), 2206-2211, (2006).
- [71] Iefuji, H., Chino, M., Kato, M., & Iimura, Y. "Raw-starch-digesting and thermostable alpha-amylase from the yeast *Cryptococcus* sp. S-2: purification, characterization, cloning and sequencing". *Biochem. J*, *318*, 989-996, (1996).
- [72] Paquet V, Croux C, Goma G, and Soucaille P, "Purification and Characterization of the Extracellular α -amylase from *Clostridium acetobutylicum* ATCC 824", *Applied and Environmental Microbiology*, *57*, 212-218, (1991).
- [73] Mukesh D.J. M Kumar, Jayanthisiddhuraj, Amutha B., Monica Devi D., Bala Kumaran M.D and Kalaichelvan P.T., "Purification and Characterization of Amylase and-Galactosidase from *Bacillus* Sp. MNJ23 Produced in a Concomitant Medium", *American-Eurasian J. Agric. & Environ. Sci.*, *12*, 566-573, (2012).
- [74] Singhania, R. R., Patel, A. K., Soccol, C. R., & Pandey, A. "Recent advances in solid-state fermentation". *Biochemical Engineering Journal*, *44* (1), 13-18, (2009).
- [75] Satoh, E., Uchimura, T., Kudo, T., & Komagata, K. "Purification, characterization, and nucleotide sequence of an intracellular maltotriose-producing alpha-amylase from *Streptococcus bovis* 148". *Applied and Environmental Microbiology*, *63* (12), 4941-4944, (1997).
- [76] Melasniemi, H. "Characterization of alpha-amylase and pullulanase activities of *Clostridium thermohydrosulfuricum*. Evidence for a novel thermostable amylase". *Biochem. J*, *246*, 193-197, (1987).
- [77] Nielsen, J.E.; Borchert, T.V., "Protein engineering of bacterial alpha-amylases". *Biochim Biophys Acta* *1543*, 253-274, (2000).
- [78] Van Der Maarel, M. J., Van Der Veen, B., Uitdehaag, J., Leemhuis, H., & Dijkhuizen, L. "Properties and applications of starch-converting enzymes of the α -amylase family". *Journal of biotechnology*, *94* (2), 137-155, (2002).
- [79] Kulp, K., Ponte Jr, J. G., & D'Appolonia, B. L. "Staling of white pan bread: Fundamental causes". *Critical Reviews in Food Science & Nutrition*, *15* (1), 1-48, (1981).
- [80] Chi, Z., Chi, Z., Liu, G., Wang, F., Ju, L., & Zhang, T. "Saccharomycopsis fibuligera and its applications in biotechnology". *Biotechnology advances*, *27* (4), 423-431, (2009).