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Review on Scaling up α-Amylase Production by Bacterial Strains through Solid State Fermentation

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Abstract

Amylases are the most important extra cellular hydrolase enzymes used in industry. An Alpha-amylase catalyzes the hydrolysis of internalα-1,4-glycosidic linkages in starch to yield monosaccharides like glucose & maltose. Alpha-amylase hydrolysis starch into simple sugars. Alpha-amylase has maximum demand for its wide application in industrial purposes for food, fermentation, paper, cosmetics, textile, pharmaceutical & biofuel industries. The world market has 248916 industrially used enzyme is nearly13,608 crore 99 lakh 20 thousand. Industries found an alternative method for the production of α -amylases from plant and microbial sources instead of chemical catalysts. This review provides a comprehensive overview of bacterial α -amylases, its production by Solid State Fermentation, and analysis of physical & chemical parameters of a-amylase production. Also this review highlights applications of α-amylases in various industries and its future perspectives.

Keywords: Alpha-amylase, SSF, Substrate, Microorganisms, DNS, Applications.

1. Introduction

Enzymes are natural catalysts that make biological processes, allowing them to be made faster and more efficiently(46). Alpha amylase, an extracellular enzyme that catalyzes the hydrolysis of internalα-1,4-glycosidic linkages in starch and similar monosaccharides by producing α– monomeric sugars and short-chain dextrin through the breakdown of internal bonds(43). Amylases are used widely in starch, detergent, beverage, and textile industries, etc… Alpha amylases are produced from plant, animal and microbial sources. The commercial production of amylase using microbes is about 25-33%. Industrial production costs for the enzymes can be feasible by using low-cost substrates such as agricultural wastes like sugarcane bagasse, corn, potato peel, rice bran, wheat, and so on. In recent studies, energy stored in agro-industrial byproducts like sugarcane bagasse has achieved increasing effort compared to other byproducts(11).

In Amylase production, Compared to bacteria, fungi can produce huge amount of α-amylases, but they are inactivated over 40°C. On the other hand, *Bacillus subtilis*, *Bacillus megatarium*, *Bacillus amyloliquefacians*, *Bacillus licheniformis*, *Bacillus stearothermophilus* produce more heat stable α-

amylases. It has been earlier reported that bacterial species produce alpha-amylases produced by microbes, that are stable at 70°C [23].

Microorganisms produce different alpha-amaylases depending on their inherent characteristics, which are thermostable and halotolerant.

Genetic engineering of the microbes is done to produce alpha-amylase with special charcteristics such as thermostability[15].The Antarctic Sea ice bacterium *Pseudoalteromonas s*p., M175 harbors a unique α-amylase gene named amy175 that is resistant to salt and cold. The gene was cloned and produced successfully [52]. Cold adapted α- amylase was produced by *Nocardiopsisaegyptia* based on the fermentation conditions [2].

For the production of Alpha amylases, Solid State Fermentation is used due to itscost efficiency with agricultural waste assubstrate. SSF is more desirable versus submerged fermentation as it gives the microorganisms their own habitat.The substrates such as molasses, husks, bagasse, seeds, corn, wheat,leaves, stem, wood shavings, sawdust, straw, stalk, shell, pulp, stubble, peel, roots, etc.. can be used[45]. SSF is a slower process as compared to SmF, because of taking the lowcostsubstrates as nutritional sources by the microbes.Most commonly *Bacillus thuringiensis* and *Bacillus cereus* engage as a co-culture condition in SSF[14].

1.1 Types of α-Amylase

1.1.1 α-Amylase:

Alpha-amylase, also known as glycoside hydrolases, is vital for breaking down starch by α-1 4 glycosidic bonds, producing glucose and dextrin.Amylose and amylopectin are the two types of polymers that form starch, a polysaccharide. In 1833, Anselme Payen discovered the first amylase[26]. As well known, alpha-amylase is an endoamylase; insidea molecule the endoamylasescatalyze hydrolysisuniquely, forming oligosaccharides with varying chain lengths that are both linear and branching. Alpha-amylase have four conserved configurations (I–IV), which are identified by Nielsen and Borchert as β-strands 3, β-strands 4, and β-strands 5 in the loop that joins β-strand 7 to α-helix 7[34]. Mostly *Bacillus sp*. and *Aspergillus sp*. produce alpha-amylases[19].From Ancient times, amylases derived from plants and microorganisms have been utilized as food additives. Brewing companies have employed barley amylases. Oriental food has long been prepared with the aid of fungalamylases[43].

1.1.2 β – Amylase:

The exo-hydrolase enzyme β-amylase produces maltose units by hydrolyzing α-1, 4-glucan linkages at the nonreducing end of a polysaccharide chain. The hydrolysis of branched polysaccharides like glycogen or amylopectin is not effective because it is unable to break the branching connections in these molecules, leaving dextrin units left behind. The ideal pH range from 4.0 to 5.5. The primary sources of β-Amylase are sweet potatoes and seeds from higher plants. It is utilized to make high-maltose syrups [41].

1.1.3 γ – Amylase:

The ideal pH for γ-Amylase is 3, and it works best in acidic conditions. Unlike other kinds of amylases, γ-Amylase cleaves not only the last α (1-4) glycosidic links at the non-reducing end of amylose and amylopectin, but also α (1-6) glycosidic linkages[41].

2. Microbial Sources

Amylolytic enzymes that digest starch, the primary polysaccharide in plants. Amylases can be obtained from plants, animals and microbes, but production from microbial sources is cost-effective and meets industrial needs [26]. Both bacteria and fungi which include molds and yeast create microbial amylases.

- Among Bacteria like *Bacillus subtilis, Bacillus licheniformis, Bacillus megatarium, Clostridium thermosulfurogenes, Aeromonas caviae, Pseudomonas sp.,*[29]*.Bacillus stearothermophilus, Geobacillus bacterium, Nesterenkonia sp. strain F, Bacterium pseudoalteromonas sp.* M175 [15] are used in amylase production.

- Among Fungi – Molds like *Aspergillus oryzae, Aspergillus kawachii, Aspergillus niger, Aspergillus awamori, Rhizopus oligosporus,Rhizopus japonicas*. And Yeast like *Saccharomycopsiscapsularia, Amylomycesrouxii*[29] are used in amylase production.

2.1 Bacterial Amylase Production by SSF

SSF is the earliest method which occurs in a solid matrix with adequate moisture (absence or near absence of free water) to support the growth of microorganisms[35]. Agro-industrial residues were selected based on a number of physical factors, including the substrate's nutrient composition, moisture content, intra-particle spacing, and particle. Agro-industrial residues such as cassava bagasse, sugarcane bagasse, sunflower oil meal, sugar beat pulp/husk, orange bagasse, oil cakes, apple pomace, grape juice, grape seed, coffee husk, wheat bran, cow dung and coir pith. Recently, cassava bagasse and sugarcane bagasse are in usage compared to other substrates because its ash content is low. Upcoming agro waste which has a potential candidate is coffee,pulp/husk because, it contains high amount of organic matter and high nutrient value.

Compared to submerged fermentation, SSF has benefits including higher volumetric productivity, less expensive equipment, higher product yields, less waste production, and fewer time-consuming procedures[6]. According to Babu and Satyanarayana, wheat bran moistened with tap water is considered to be the best substrate for the alpha amylase production by *Bacillus coagulans*[5]

Alpha-amylaseproduced by *Bacillus subtilis*usesBanana peel as a substrate using Solid State Fermentation method (SSF) at an incubation period of35°C for 24 hrs, substrate concentration as 50g, pH 7, Since alpha-amylase is an extracellular enzyme. Enzyme assay was performed by spectrophotometric method using culture filtrate and 4-10 fold increased production was noted in comparison to wheat bran and rice bran [49].
An extracellular enzyme of moderately halophilic *Bacillus cereus* isisolated from salt pan soil using black gram husk, green gram husk, sugarcane bagasse, wheat bran by SSF method. Maximum yield of amylase production was obtained with sugarcane bagasse at 40°C for 48 hrs on pH 7,Yeast extract as nitrogen source andMaltose as carbon source, Lactose as a supplementary carbon source[51].

A thermostable extracellular alpha amylase was produced by *Paenbacillusamylolyticus*by SSF using wheat branmoistened with a solution ofNutrient broth (10g), Soluble starch (10g), lactose (5g), NaCl (5g), CaCl2 (2g)with aoptimumpH 8.0 and incubated at37°C for 72 hrs. Enzyme extraction was done using 0.02M phosphate buffer. Enzyme Assay was carried out by estimating reducing sugars which is quantified with Somogyi method. The obtained α-amylase production is 275.95±2.75 U/g/minwhen 10 g of wheat bran moistened with 10ml (M2) of dilutent[24].

*Bacillus circulans ATCC 4516*produces alpha-amylase using rice bran as a solid substrate producing 2716.9 ± 35.9 U/mg of enzyme. The optimized parameters include pH 7.5 at 37°Cfor 48 hours also supplemented with Ammonium Chloride as nitrogen source which increases the enzyme production.Enzyme assay was carried out by Bernfeld method using soluble starch as a substrate[42].

*Bacillus licheniformis*producesathermophilic & alkaliphilic alpha-amylase. Maximum enzyme production was obtained using beef extract (3g), Peptone (5g), Starch (1%). 0.15% of peptone include pH 8 at 50°C for 48 hours. Enzyme assay was carried out by Spectrophotometric method and DNSmethod[22].

2.2 Substrate

2.2.1 Moisture

Moisture has an important role in the production of amylase production for the growth of the microorganism. Low or High level of moisture content will affect the enzyme activity. Basically, Bacteria would grow on optimum moisture level 70-80%[46]. *Thermomyceslanuginosus* ATCC 58157 gives maximum amylase production when moisture of wheat bran is adjusted to 90%[27]. *Bacillus Coagulans* showed maximum amylase production on 65% moisture in wheat bran[32].

2.2.2 Carbon Source

The most commonly used carbon sources include starch, molasses, glycerol, fructose, glucose and its derivatives, maltose, sucrose, lactose, dextrin, cyclodextrin, glycogen, cellulose, and hemicellulose. It shows maximum yield of enzyme production when galactose, glucose and inulin were added in the medium *Bacillus sp.* and particularly, *Bacillus licheniformis*[9].If maltodextrin supplemented to Alpha- amylase medium it shows high yields of production, observed on *Thermomyceslanuginosus*[33].
Besides glucose, supplemented along with cheese whey and orange peel result in high yield of amylase by *Bacillus amyloliquefaciens*[50].

2.2.3 Nitrogen Source

Production of amylase uses either organic or inorganic sources. Organic sources such as Peptone, Yeast extract, Meat extract, Soybean, Gelatin, Casein, Cotton seed meal, Corn steep liquor are employed. Inorganic sources like Ammonium Chloride (NH4CL), Ammonium sulfate (NH4)2SO4, Ammonium nitrate (NH4N03), Sodium nitrate (NaNO3), Potassium nitrate (KNO3), Urea, Ammonia (NH3)also used[46].

Under vigorous shaking conditions, both the species of *Bacillus stearothermophilus* and*Bacillus amylolyticus*produce a high yield when the culture medium is enhanced with 1% peptone and 0.5% yeast extract[12].*Bacillus amyloliquefician*s showed maximum yield on 0.2g of Ammonium sulfateaddition[36]. P.V. Dharani Aiyer's findings indicated that Ammonium hydrogen phosphate is a superior nitrogen source compared to peptone for the production of amylase by *Bacillus licheniformis*[3].

2.2.4 Metal Ions

Addition of salt of metal ions like Calcium chloride, Magnesium, Manganese, Cobalt, Zinc, Copper, Potassium, Sodium is known to increase alpha-amylase better. The concentration varies according to the microorganisms[43].*Bacillus amyloliquefician*s shows maximum yield when CaCl2 is used[54].Alpha-amylasefrom *Bacillus sp.,* ANT-6 exhibits enhanced alpha-amylase production when 5mM CaCl2 (calcium chloride) and PMSF (phenylmethylsulfonyl fluoride) are added[8].

2.3 Process Parameters

2.3.1 pHand Temperature

pH is the negative logarithm of hydrogen ion concentration. Enzymes are sensitive to pH, they are active only at specific pH. Alpha-amylase are extracellular enzymes, i.e, they are secretedoutside the cells hence, the medium pH plays an important role and thus the optimal pH for bacterial alpha amylases 5.5 to 7.0[46]. Study on *Bacillus sp.* showed pH 7.0 after 30 min with 1% of starch in 0.05M PBS buffer [28]. *Rhodothermus marinus* improved results at a pH of 7.5 to 8. The optimal pH for maximum enzyme production varies among different species. The ideal temperature range for the production of bacterial alpha-amylase is between 30-45°C.

In *Bacillus licheniformis*showed high yield when incubated at 60°C for 10 mins,but at 80°C it shows decrease in amylase production[31].*Bacillus megatarium*shows maximum production at 35°C pH 7.5 and minimum production at 50°C pH 8.5[39].*Bacillus amyloliqufecians*, gives high yieldsalpha-amylase production at 37°Cin the presence of mannitol and D-inositol [37].

2.3.2 Fermentation Period

Time duration in fermentation process is the most critical factor. Increase or decrease of incubation period leads to loss of enzyme production, because due torelease of toxic substance or depletion of nutrient in the medium.*Bacillusaxarquiensis*and *Bacillus subtilis*, showed maximum growth at 48h incubation period & maximum enzyme production at64.5 U/ml and 345 U/grespectively[4,21].

3. Enzymatic Activity Assay

An enzymatic activity assay determines how an alpha-amylase can convertstarch into smaller sugars. The various techniques includesDinitrosalicylic Acid Method (DNS),Nelson – Somogyi (NS) Method,Determination of Activity using Iodine,Reduction in Viscosity of Starch Suspension.

3.1 Dinitrosalicyclic Acid Method (DNS)

Dinitrosalicyclic Acid Method (DNS) is commonly used to quantify reducing sugars such as maltose, and glucose. To the crude enzyme extract starch solution was added and incubated at 50°C for 10 mins. During this, the starch is broken down by the enzyme into reducing sugars. After incubation, add 2ml of

DNS reagent to the test tube to stop the reaction. Incubate the tube in a boiling water bath for 5 minutes thus the reducing sugar reacts with it to form a reddish-brown complex. After cooling at room temperature, the absorbance of the supernatant is measured at 540nm using a spectrophotometer. By using a standard curve, can determine the concentration of reducingsugars[7]. Enzyme obtained from *Bacillus amyloliqufecians*, estimated with glucose(Standard), 0.75 mL of 0.1 M acetate buffer (pH=5.0) and 1.25 mL of1 % starch solution (blank) was read at 510nm using a spectrophotometer. Maximum activity was found to be 62,470 U/g at 72h[16].

3.2 Nelson– Somogyi (NS) Method

Nelson-Somogyi method is a biochemical assay used to estimate the reducing sugars. A stock solution is heated at 50°C for 5min. Preheated crude enzyme extractwas added to the substrate. The reaction mixture is incubated at 50°C for 10 min. After incubation, to terminate the reaction somogyi copper reagent is added. Followed by this keep on boiling water bath for 40 min. After cooling at room temperature, add Nelson arsenomolybdate reagent and incubate at room temperature for 10 min. Add water to the mixture and centrifuged at 13,000rpm for 1min. The absorbance of the supernatant is read at 610 nm using a spectrophotometer[7].*Geobacillusicigianus BITSNS038* produces alpha-amylase were estimated by NS method,Maltose was measured at 620 nm using a spectrophotometer. After incubation of 18h, maximum enzymatic activity was found to be 2.983 U/ml also after 24h, they observed that enzymatic activity gradually decreasedup to 0.349 U/ml at 72h[44].

4. Purification of α-Amylase

On Industrial applications and commercial purposescrude enzymes and moderate downstream processing were required. Forthe pharmaceutical and clinical industry Alpha amylase was found to be in pure form.Also forthe study of structural characteristics, functional, physical and biochemical properties require a purified form of the enzyme.The most commonapplied methods are precipitation, and chromatographic techniques such as affinity, gel-filtration, ion exchange, dialysis, liquid-liquid extraction, and Ultrafiltration[20]. An Alpha-amylase obtained from *Bacillus licheniformis*were performed an anion-exchange chromatography and gel filtration column. They were pooled together to perform dialysis under ultrafiltration. As a result, initial alpha-amylase activity was found to be 4390 U/mg. After purification, the enzyme activity was reduced upto 749 U/mg[1].Purification on *Thermomonosporacurvata*, the crude enzyme was purified by Amicon model TCF-10 ultrafiltration. Ethanol precipitation protocol wasperformed by adjusting pH. The precipitate was subjected into Sephadex G-150 column and fractions were performed bydiethylaminoethyl cellulose (DEAE). Fractions were pooled and concentrated on an ultrafiltration unit using a membrane filter. The resultant activity was 9% of 66-fold purification gained from the initial enzymatic activity. Hence, after purification, the enzymatic activity wasincreased[17].

5. Characterization of α-Amylase

Once an enzyme is purified, characterization of an enzyme should be performed. It determines the hydrolysates observed after an enzyme action on starch by PAGE. The purified enzyme was performed with molecular markers like BSA, ovalbumin was run on the gel. The resultant bands were obtained. These bands were stained with coomassive brilliant blue and Silver nitrate for a clear visualization. Commonly used characterization methods were SDS-PAGE, Zymography, NMR Spectroscopy[46].

Alpha-amylase obtained by *Bacillus licheniformis*wereestimatedby SDS-PAGE with Silver nitrate revealed to be 55kDa[1].*Streptococcus bovis*148produces intracellular alpha-amylasewhich produced a single band on SDS-PAGE and gel filtration showsa molecular mass of 57,000 Daand 55,000 Da respectively. Thus, they are clearly in monomeric form[40].

6. Industrial Application of α-Amylase

6.1 Industrial Production of Starch

The starch industry hasone of the most important application of alpha-amylase by which starch hydrolysis converts starch into fructose and glucose for the production of syrups[46]. It requiresthree steps for syrup productionfirstly, Gelatinization:the granules of starch (amylase and amylopectin)are disintegrated, and dissolved in water to forma viscous starch solution.Secondly, Liquefaction: partial hydrolysis of α -amylase into short-chain dextrin gives the reduced form of viscosity in starch solution. Thirdly, Saccharification:glucoamylase, acting as an exo-amylase, cleaves α-1,4 glycosidic linkages from the non-reducing terminal end. For glucose syrups, the action of pullulanase with glucoamylase takes place. To produce fructose syrup, high-yield glucose is converted into fructose syrup via glucose isomerase, which is catalyzed by isomerization. This process requires thermostable α-amylase to reduce processing time and the risk of contamination.*Bacillus Stearothermophilus, Bacillus amyloliqufecians,Bacillus licheniformis and Pyrococcusfuriosus*produce alpha-amylase whichare used in starch industries for the conversion[30].

6.2 In Detergent Industry

The primary consumers of enzymes are the detergent industries. Nearly 90% of detergents have enzymes in their formulation which are eco-friendly compared to chemical detergents.which are eco-friendly compared to chemical detergents. Most commonly *Aspergillus sp. and Bacillus sp*produce alpha-amylase, used for detergent production [30]. Clothes stained with food particles and soil particlescontainingstarch are broken down into smaller water-soluble oligosaccharides by a- amylase. Thus, eliminating starch is crucial to keep clothing white. The common use of a-amylase in detergents can be attributed to its stability at low temperatures and alkaline pH levels [46].

6.3 In Paper Industry

The paper industry has a significant role in utilizing α-amylase enzymes. Paper and Pulpindustry uses alpha-amylase to coat the surface of the paper for better quality.Starch is a good sizing agent. Cold active alpha-amylase is used due to its reduction on viscosity when coated on paper[20]. The embrittlement of starch paste, used as a mounting adhesive and often modified along with alum or protein glue, canresult in damage to paper to address this issue, α-amylase is applied as a gel poultice or in immersion [38].

6.4 In Textile Industry

During the weaving of clothes, the starch paste is wrapped to promote the fabric strength of the clothes. Similarly, starch is used as a coating agent in the paper industry. After weaving of cloth, starch has been removed. *Bacillus sp*. produces alpha-amylasecanbe employed in the field of textile industry[30]. To remove starch from grey cloth, additional processing such as bleaching and dyeing is required. Alpha-

amylase is used as a desizing agent to facilitate this process. After mashing process, some clothes may shrink. Finally, the desired clothes are cleaned and laundered [38].

6.5 In Food Industry

Alpha-amylase is primarily used in the dough process of the bread-baking industry to increase the rate of fermentation. This enables the starch to hydrolyze into dextrin, facilitating faster yeast fermentation. However, after the baking process, bread is susceptible to spoilage, including increased crumb firmness, loss of crust crispness, reduced moisture content, and diminished bread flavor. These changes are collectively referred to as staling. Alpha-amylase not only promotes fermentation but also extends the shelf-life of baked products and improves their softness retention, earning it the designation of anti-staling agent. To overcome the challenges posed by staling, alpha-amylase is employed[20,25].

6.6 In the field of Biofuel production

Ethanol is the most commonly used biofuel. Its production involves liquefaction to solubilize starch, followed by saccharification, a process that converts starch into sugar using amylolytic enzymes or exoenzymes. Subsequently, yeast such as *Saccharomyces cerevisiae* convert the sugar into ethanol [48]. Recent advancements in biotechnology have led to the development of novel yeast strains through protoplast fusion. This technique was employed to fuse amylolytic yeast, such as Saccharomyces cerevisiae and *Saccharomyces fibuligera*, resulting in a novel strain capable of directly producing ethanol from starch, thereby eliminating the need for a separate saccharification process [10].

6.7 In the field of clinical and pharmaceutical industry

The pharmaceutical industry utilizes pure forms of alpha-amylase in their products. Primarily, it addresses digestive aid problems due to its effective digestive properties, which facilitate easy digestion of food in the body. Enzyme therapy offers a significant advantage over traditional treatments, as it targets the root cause rather than just alleviating symptoms. This approach is safe, easy to use, and free of side effects [13]. Adenosine deaminase (ADA) was the first clinical gene therapy treatment, aimed at curing an enzyme deficiency. Research has shown that patients with chronic pain correlate their salivary gland alpha-amylase activity and pain levels. The Visual Analogue Scale (VAS) is commonly used to assess pain severity and develop treatment plans [53]. Medications incorporating alpha-amylase are used to treat various digestive disorders, including pancreatic exocrine insufficiency (PEI), pancreatitis, cystic fibrosis, and diabetes types I and II. *Bacillus species*, particularly *Bacillus stearothermophilus* and *Bacillus licheniformis*, produce amylases, which can be combined with lipase and protease for medicinal applications [47].

7. Conclusion

This review highlights the significance of alpha-amylase, an industrially important enzyme produced by microorganisms, which has yielded reliable results in research. The Solid-State Fermentation (SSF) method is predominantly one of the method used for production due to its cost-effectiveness, requiring only low moisture content, easily available substrates, and resulting in high enzyme yields. Although numerous microbial sources can produce alpha-amylase, only a few strains are utilized for industrial purposes. Alpha-amylase has extensive applications due to its biodegradable nature, making it environmentally safe and clean. Ongoing research focuses on utilizing alpha-amylase to treat type 2 diabetes, as well as investigating plant-derived inhibitors to study their characteristics. Researchers

continue to explore plant-derived compounds for potential anti-diabetic medications. Alpha-amylase has diverse applications in various industries, including textiles, paper, chocolate, bread, brewing, and biofuel production. In the pharmaceutical industry, alpha-amylase presents emerging hope for new discoveries and opportunities in the coming years.

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