

**Opinion**

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# Acidic $\alpha$ -amylase of *Bacillus acidicola*: Attempts in Ameliorating Catalytic Efficiency and Production



**T Satyanarayana\***

Division of Biological Sciences & Engineering, Netaji Subhas Institute of Technology, New Delhi, India

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**\*Corresponding author:** T Satyanarayana, Division of Biological Sciences & Engineering, Netaji Subhas Institute of Technology, Azad Hind Fauji Marg, Sector-3, Dwarka, New Delhi-110078, India, Email: [tsnarayana@gmail.com](mailto:tsnarayana@gmail.com)

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## Opinion

$\alpha$ -Amylases are widely used enzymes in starch saccharification and baking industries, and in textile desizing. These are endo-enzymes which cleave  $\alpha$ -1,4-glycosidic linkages randomly in starch, thus, generate maltose and malto oligosaccharides. The commercially available  $\alpha$ -amylases have certain limitations like a limited activity at low pH and  $\text{Ca}^{2+}$ -dependence, therefore, the search for novel acid stable, thermostable and  $\text{Ca}^{2+}$ -independent amylases from extremophilic microorganisms has been a major area of research over the years.  $\alpha$ -Amylase with the twin characteristics of thermostability and acid stability, to the extent desired, has not been easy to generate. We have been able to lay our hands on an acidophilic bacterium that produces acid stable  $\alpha$ -amylase. By extensive efforts, we have successfully generated a recombinant acid stable and  $\text{Ca}^{2+}$ -independent  $\alpha$ -amylase and produced it in high titres.

Starch is the second major food reserve polysaccharide in nature after cellulose. Plants are unique in synthesizing this  $\alpha$ -glucan that serves as an important source of nutrition for other living organisms. Starch is the most easily available source of carbon and energy on Earth and is synthesized by plants in the presence of sunlight and water through photosynthesis. Starch is synthesized in plastids present in leaves and accumulated as insoluble granules in higher and lower plants, and is synthesized as semi-crystalline granules with different polymorphic types and degrees of crystallinity. The granule size varies from 2 to 100  $\mu\text{m}$  with round, oval and irregular shapes. Starch is a major component of most of the staple foods and is used in many food and non-food industries.

The structural organization of starch is mainly composed of two high molecular weight compounds amylose and amylopectin and both these contain  $\alpha$ -D glucose as a sole monomer. Amylose is a linear water insoluble polymer of glucose subunits joined by  $\alpha$ -1, 4 glycosidic bonds (99%) with the molecular weight of  $\sim 1 \times 10^5$  to  $1 \times 10^6$ . On the other hand, amylopectin is branched water soluble polysaccharide with short  $\alpha$ -1, 4 linked ( $\sim 95\%$ ) linear chains of 10-60 glucose units and  $\alpha$ -1, 6 linked ( $\sim 5\%$ ) side chains with 15-45 glucose units that forms the volume of starch molecule. The ratio of amylose to amylopectin varies in starches, but representative levels of amylose to amylopectin are 25-28% and 72-75%, respectively.

The starch is mainly consumed after processing for domestic or industrial purposes. Starch hydrolyzed products have applications in food, beverage and pharmaceutical industries. Till 19<sup>th</sup> century, acid hydrolysis using dilute HCl was carried out for starch saccharification, because the understanding of the potential advantages of biological catalysts was limited. The enzymatic starch processing has advantage over chemical starch hydrolysis as the latter has demerits like high temperature and low pH requirement, low glucose yields, formation of unwanted color, bitter tasting compounds, and the need for corrosion resistant vessels. Today starch saccharification is totally enzyme based.

The world market for industrial enzymes was estimated at US\$5.1 billion in 2009. Acid-stable extracellular enzymes are required as they have applications in the degradation of polymeric or oligomeric carbon sources, the pH of which lie

between 3.2 and 4.5. The promising properties of enzymes from thermoacidophiles are anticipated to be active at low pH and elevated temperatures, therefore, these can be used in starch and textile industrial processes and in fruit juice industry. The demand for enzymes from extremophiles may increase in future since they are active under harsh industrial process conditions. A variety of polysaccharide hydrolyzing enzymes suited for various industrial applications have emerged in the last few decades that led to screening of enzymes for novel properties [1-4].

### Starch saccharifying enzymes

Amyolytic enzymes ( $\alpha$ -glucanases) hydrolyze glycosidic linkages in various  $\alpha$ -glucans. They belong to mainly 3 families of glycoside hydrolases (GHs), GH 13 (the  $\alpha$ -amylase family), GH 14 ( $\beta$ -amylases), and GH 15 (glucoamylases). The enzymes differ in amino acid sequences, reaction mechanisms, catalytic activities and structural characteristics. Based on the mode of action, the enzymes have been divided into two categories: endoamylases ( $\alpha$ -amylases, pullulanases, isoamylase) and exoamylases ( $\beta$ -amylase, glucoamylase). The dextrinogenic or liquefying amylases (endoamylases) act randomly on  $\alpha$ -1,4-linkages only.  $\alpha$ -Amylases are extracellular enzymes, which catalyze the hydrolysis of  $\alpha$ -1,4 glycosidic linkages in starch liberating linear and branched oligosaccharides of varying chain lengths and glucose. These are categorized on the basis of end product formation as maltose-forming (eg. *Bacillus acidicola*), maltotetraose-forming (*Pseudomonas* sp. IMD 353), maltopentaose-forming (*B. cereus* NY-14), and maltohexaose-forming (*B. stearothermophilus* US100)  $\alpha$ -amylases.  $\alpha$ -Amylase catalyses hydrolysis of (1-4)- $\alpha$ -D-glucosidic linkages in polysaccharides and successively removes  $\alpha$ -maltose, maltotetraose, maltopentaose and maltohexaose from the non-reducing ends of starch. Liquefying  $\alpha$ -amylases carry out the rapid reduction in viscosity of starch pastes without producing free sugars. On the contrary, saccharifying  $\alpha$ -amylases produce free sugars but reduce the viscosity slowly as compared to liquefying  $\alpha$ -amylases. The search for  $\alpha$ -amylases with the desired kinetic properties for diverse applications is encouraged, because these will improve the industrial process in terms of economics and feasibility. Based on the pH for activity, acidic, neutral and alkaline  $\alpha$ -amylases are also known. The pH optima of  $\alpha$ -amylases vary in the range between 2.0 and 12.0.  $\alpha$ -Amylase from *Bacillus subtilis*, *B. licheniformis*, *Micromonospora melanospora*, and *Geobacillus thermoleovorans* display highest activity at pH 6.0, 6.5, 7.0 and 8.0, respectively. Presently, amylases have the major world market share of enzymes. Bacterial amylases are generally preferred over fungal amylases due to characteristic advantages that the former offer. Acidstable  $\alpha$ -amylases will be preferred as their application minimizes contamination risk too.

$\alpha$ -Amylase is one of the most important industrial enzymes employed in the starch processing industry for the production of starch hydrolysates. The pH of native starch is 3.2-4.5, and

therefore, thermostable acidic and  $\text{Ca}^{2+}$ -independent  $\alpha$ -amylases suit better in the conversion of starch into various sugar syrups. Acidic  $\alpha$ -amylases are known to be produced by bacteria, archaea and fungi. These are also used in the removal of starch in beer, fruit juices, and from textiles and porcelain. The maltogenic amylase is used as an antistaling agent in order to prevent the retrogradation of starch in bakery products. Amylases are also gaining importance in biopharmaceutical applications. Their application in food and starch based industries is the major market, and the demand for  $\alpha$ -amylases has been rising continuously.

### Acid-stable $\alpha$ -amylases

The demand for high maltose-forming  $\alpha$ -amylases has been increasing as these have diverse commercial applications. The  $\alpha$ -amylases currently used in starch processing are active at 95 °C and pH 6.8, and stabilized by  $\text{Ca}^{2+}$ , therefore, the process cannot be performed at low pH (3.2-4.5), the pH of the native starch. In order to be compatible with the pH optima of the enzyme used in liquefaction, the pH of the starch slurry is raised from its native pH 3.2-4.5 to 5.8-6.2, and further,  $\text{Ca}^{2+}$  is added to enhance the activity and/or stability of enzyme. The next saccharification step again requires pH adjustment to pH 4.2-4.5. Both these steps (adjustment of pH and removal of salts) need to be omitted, as they are time consuming and add to the cost of the products. The emphasis is, therefore, on extremozymes from extremophiles that are naturally endowed with the properties required for specialized industrial applications. Several fungi (*Aspergillus hennebergi*, *A. usamii*, *A. foetidus*, *Thermomyces lanuginosus*), bacteria (*Bacillus acidocaldarius*, *B. circulans*, *B. caldolyticus*) and archaea (*Pyrococcus furiosus*) are known to produce  $\alpha$ -amylases which are active in the acidic pH range between 3.0 and 6.0. A point to be noted is that highly acidic amylases are not adequately thermostable, and those that are highly thermostable are not adequately acid-stable.

### Our search for acid-stable $\alpha$ -amylase

In our search for acid-stable  $\alpha$ -amylases, we have isolated bacteria from various environmental samples, screened and selected a bacterial strain identified as *Bacillus acidicola* that produces acidic and moderately thermostable  $\alpha$ -amylase. Our extensive and laborious efforts led to optimize enzyme production in submerged fermentation to 10,000 units per litre. The pure acidic  $\alpha$ -amylase of *B. acidicola* is optimally active at pH 4.0 and 60 °C with a T<sub>1/2</sub> of 27 min at 90 °C.

### Cloning and expression acidic $\alpha$ -amylase gene

$\alpha$ -Amylase was one of the first proteins adopted for molecular biological studies because of many reasons such as the existence of easy screening assay, availability of amylase negative strains, knowledge of genetics, protein production and fermentation technology of  $\alpha$ -amylases. The  $\alpha$ -amylase encoding gene of *B. acidicola* with N and C terminal truncation has been cloned in pET28a (+) and expressed in *E. coli*. The

recombinant *E. coli* produces 15-fold higher amylase than the native strain. The recombinant amylase is intracellular in *E. coli* which needs energy intensive cell disruption methods for its release. The 62kDa recombinant  $\alpha$ -amylase is optimally active at pH 4.0 and 60 °C, like the native enzyme. The molecular mass of native  $\alpha$ -amylase of *B. acidicola* is 66kDa, which is higher than that of the recombinant amylase, confirming that the former is truncated.

The chimeric  $\alpha$ -amylase was generated by adding 37 amino acids to the N-terminal and 11 to C-terminal ends of the truncated recombinant  $\alpha$ -amylase (479aa) from that of *Geobacillus thermoleovorans*. The chimeric  $\alpha$ -amylase expressed in *E. coli* consisted of 527 amino acids with a molecular mass of 68kDa. This displays a marked increase in catalytic efficiency and thermostability. The recombinant chimeric  $\alpha$ -amylase gene was also cloned and expressed extracellularly in *Pichia pastoris*. A high enzyme production (750Uml<sup>-1</sup>) was achieved by high cell density fed batch fermentation. The molecular mass of the glycosylated recombinant chimeric amylase is of 75kDa with T1/2 of 40min at 90°C, which is higher than that of the native amylase. These molecular approaches aided in improving production, thermostability as well as catalytic efficiency. Thermostability of the chimeric amylase is inadequate for its application in the conventional starch saccharification, although it has the desirable characteristics of acid-stability and Ca<sup>2+</sup>-independence. We have also generated a chimeric  $\alpha$ -amylase-glucoamylase by linking both enzymes using a linker peptide of 25 amino acids, which converts starch into glucose in one step.

Further efforts are, therefore, called for finding  $\alpha$ -amylase with the desirable characteristics of acid-stability, thermostability and Ca<sup>2+</sup>-independence by isolating bacteria and archaea from acidic and hot environments like acidic hot springs, acidic soils, acid mine drainage and others. Attempts would also be needed to retrieve genes that encode acid-stable  $\alpha$ -amylases

from environmental samples through culture-independent metagenomic approaches. Extensive efforts would be needed for improving production levels to bring down the cost of enzyme production and ameliorating properties to suit the targeted applications [5-9].

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