Microbial α-Amylases in the Industrial Extremozymes

Annisyia Zarina Putri1 and Tomoyuki Nakagawa1,2*

1 The United Graduate School of Agricultural Science, Gifu University, Tokai National Higher Education and Research System, 1-1 Yanagido, Gifu, 501-1193, Japan
2 Faculty of Applied Biological Sciences, Gifu University, Tokai National Higher Education and Research System, 1-1 Yanagido, Gifu, 501-1193, Japan

ABSTRACT

Amylase is part of an enzyme group that hydrolyzes starch, and many amylases are commercially used for starch hydrolysis in several industrial processes. For industrial applications of amylases, the reaction conditions for starch hydrolysis also differ depending on each industrial use. There are various microbial amylases that have been isolated and developed, which have suitable enzymatic properties for each application. Especially, some microbial amylases included in "extremozymes" have been widely applied to each industrial process. The purpose of this review is to summarize the classification of amylases and the diversity of microbial α-amylases and their current industrial applications, and we would like to indicate the direction of research in screening and applications for novel types of amylases included in "extremozymes".

Keywords

amylases, extremozymes, industrial enzymes, starch

1. Introduction

Starch is a polymeric carbohydrate that consists of numerous glucose units. Starch contains two types of α-glucans, i.e., amylose and amylopectin. Amylose is a linear water-insoluble polymer, which is a glucose polymer joined by 1,4-α-D-glycosidic bonds (Fig. 1A), while amylopectin is a branched polysaccharide with short α-1,4 linked linear amylose chains and α-1,6 linked side chains with glucose units that form the volume of starch molecule (Fig. 1B) (Buleon et al., 1998; Tester et al., 2004). Starch is a major storage product of many economically important crops, such as rice, wheat, potato, cassava, and other many crops, and it is one of the most important energy sources for human nutrition (van der Maarel et al., 2002). Therefore, most of the world’s population either directly uses starch or derives food and energy from its hydrolysates (van der Maarel et al., 2002).

On the other hand, enzymes are biological catalysts that are a critical component of biological reactions in the cell, and several kinds of enzymes such as proteases, lipases, and amylases are used in several industrial fields, such as in detergents and in the paper industry, textile industry, food industry, and in many others industrial applications. These enzymes are called “industrial enzymes”, and microbial enzymes are mainly applied to these industrial processes. The industrial enzyme market has been projected to reach US$6.2 billion by 2020 (Mehta and Satyanarayana, 2016), and the market is expected to continue to grow in the future because of advances in the biotechnology industry, the continued need for cost-effective manufacturing processes, and the need for greener technologies (Sarmiento et al., 2015).
Among these industrial enzymes, amylases, which are an enzyme group that hydrolyzes starch, are one of the most frequently used groups of enzymes, and amylases comprise 30% of the world’s enzyme consumption because the starch industry is one of the largest users of industrial enzymes (van der Maarel et al., 2002). In starch-related industries, use of amylase is the most desirable method for starch degradation because it has many advantages such as improved yield and favorable economics (Satyanarayana et al., 2004). Therefore, many amylases are available commercially, and they have almost completely replaced chemical starch hydrolysis (Aiyer, 2005). Starch is currently enzymatically processed into a variety of different products, such as syrups, oligosaccharides, dextrin, thickener, stabilizer, and gelling agents. However, the reaction conditions for starch hydrolysis are different in each industrial application. Therefore, industrial enzymes sometimes need to possess extraordinary properties of stability and adaptivity for extreme temperatures, pH, and other conditions. In these cases, “extremozymes”, which are derived from the extremophiles (Hough and Danson, 1999), have high potential as industrial enzymes. On the other hand, required enzymatic properties also differ for each industrial application. For example, some starch hydrolysates were recently shown to have various food functionalities, and the demand for these hydrolysates is increasing; e.g., maltopentaose has been used as nutrient food for patients with renal failure and those in a state of calorie deprivation. Maltotetraose is also being examined as a food additive to improve texture and moisture retention in food. Therefore, novel α-amylases, which can produce the desired products in a one-step reaction from starch, are in great demand (Kandra, 2003). Other developments in the pharmaceutical and chemical industries led to extension
of its applications into novel areas such as therapeutic applications in cancer and wound healing. Therefore, various microbial amylases with suitable enzymatic properties for each application have been isolated and developed.

In this review, we summarize the classification of amylases and their diversity. In the second half, we focus on microbial α-amylases and describe microbial α-amylases in extremozymes with various enzymatic properties with their applications. Finally, we conclude the direction of research in screening of novel types of amylases included in "extremozymes" and in their applications as industrial enzymes.

2. Classification of amylases and their starch hydrolyzing process

The enzymic hydrolysis of starch is catalyzed by several types of amylases. Using several combinations of amylases such as endo-, exo-, and debranching types, all living things, including amylolytic microorganisms, use starch as their carbon source. These amylases are then applied to several industrial processes according to their specific enzymatic properties.

2.1 α-Amylase

α-Amylase (1,4-alpha-D-glucan-glucanohydrolase, EC 3.2.1.1) is an extracellular enzyme that is widely distributed in animals, plants, and microbes (Pandey et al., 2000; Kandra et al., 2003). As shown in Fig. 2, α-amylase catalyzes degradation of α-1,4-glucosidic linkage, but could not cleave α-1,6-linkages, and it breaks down long-chain saccharides by acting at random locations along the starch chain in an endo fashion (Gupta et al., 2003; Kandra et al., 2003; Rajagopalan and Krishnan, 2008).

In the starch degradation step, α-amylase has important roles for reducing viscosity and the starch chain molecular weight, and an increase in the number of starch chain molecules. Therefore, microbial α-amylases are used in various industries such as producing isomerized sugar, bakery applications, and textile de-sizing; it is also used in the paper industry and has other industrial uses.

α-Amylase is a glycoside hydrolase (GH), and GHs are classified into 167 families based on sequence similarity (CAZy, 2020), and almost all α-amylases are classified into GH family 13, which is the largest of the GH families, although some of them belong to families 57 and 119 (Cantarel et al., 2009).

2.2 β-Amylase

β-Amylase (1,4-alpha-D-glucan-maltohydrolase, EC 3.2.1.2) is an extracellular enzyme (French, 1960). β-Amylase is a major protein on the starchy endosperm of ungerminated barley seeds (Hejgaard and Boisen, 1980), and β-amylase had been thought to be distributed only in higher plants, such as barley, soybean, and sweet potato (Balls et al., 1948; Manners, 1962; French, 1960), because β-amylase was found in malts in 1924. In 1974, however,
A new type of β-amylase was found in the Gram-positive bacteria *Bacillus megaterium* (Higashihara and Okada, 1974), and many β-amylases from various bacteria and fungi were subsequently reported (Pandey *et al*., 2000; Ray, 2004).

As shown in Fig. 2, β-amylase catalyzes degradation of α-1,4-glucosidic linkage, but could not cleave α-1,6-linkages, which is the same as α-amylase. β-Amylase, however, releases successive maltose units from the non-reducing ends from the starch chains (French, 1960). Therefore, in industrial applications, β-amylase is used for fermentation in brewing and distilling industry and production of high maltose syrups.

β-Amylases are classified into GH family 14 (Cantarel *et al*., 2009).

### 2.3 Glucoamylase (γ-Amylase)

Glucoamylase (γ-amylase:1,4-alpha-D-glucan-glucohydrolase, EC 3.2.1.3) is an extracellular enzyme that is widely distributed in animals and microbes such as bacteria and fungi (Pandey *et al*., 2000). In animals, glucoamylase is located on the brush border of the small intestine, and in fungi, the enzyme is anchored on the cell wall.

As shown in Fig. 2, glucoamylase also catalyzes degradation of mainly 1,4-α-D-glucosidic linkage, similar to α- and β-amylase, but it releases successive β-D-glucose units from the non-reducing ends from the starch chains (Sauer *et al*., 2000; Tateno *et al*., 2007). Most forms of glucoamylase are able to hydrolyze 1,6-α-D-glucosidic bonds rapidly, when the next bond in the saccharide sequence is 1,4-α-D-glucosidic bond. Moreover, some glucoamylases also hydrolyze 1,6- and 1,3-α-D-glucosidic bonds in other polysaccharides. This entry covers all such enzymes acting on polysaccharides more rapidly than on oligosaccharides (Stuart, 1978).

Glucoamylase has wide diversities, *i.e.*, glucoamylases from fungi, and bacteria belong to different GH families, such as families 15, and 97, respectively (Cantarel *et al*., 2009). Glucoamylase from human intestine (EC 3.2.1.20), which belongs to GH family 31, also hydrolyzes polysaccharides, catalyzing the reactions of EC 3.2.1.3 (CAZy, 2020).

The major application of glucoamylase is the saccharification of partially processed starch/dextrin to glucose, which is an essential substrate for numerous fermentation processes and a range of food and beverage industries (Kumar and Satyanarayana, 2009).

### 2.4 Isoamylase

Isoamylase (1,6-alpha-D-glucan-glucahydrolase, EC 3.2.1.68) is an extracellular enzyme that is produced by both plants and microorganisms. As shown in Fig. 2, isoamylase is a debranching enzyme of amylopectin, and it
catalyzes the degradation of α-1,6-glucosidic branch linkage in amylopectin to yield amylose and oligosaccharides (Yokobayashi et al., 1970; McCleary et al., 2014). Isoamylases are classified into GH family 13 (Cantarel et al., 2009).

Isoamylase is used primarily in the production of food ingredients from starch, such as glucose syrup, maltose, maltitol, trehalose, cyclodextrin, and resistant starch.

2.5 Starch hydrolyzing process using amylases

All living things use sets of amylases in the biological metabolism of carbohydrates such as starch and glycogen (Poonam and Dalel, 1995). In the first step, branched starch, such as amylopectin, is debranched by isoamylase, yielding amylose. Amylose is then hydrolyzed into smaller oligosaccharides in an endo fashion by α-amylase (Gupta et al., 2003; Kandra et al., 2003; Rajagopalan and Krishnan, 2008). Finally, oligosaccharides are broken down into glucose or maltose by glucoamylase (or β-amylase), and organisms absorb glucose or maltose into the cell to metabolize them (Fig. 2). This starch hydrolyzing process is called as “starch saccharification”.

Hydrolyzed starch has applications in several industries such as the food, beverage, pharmaceutical, textile, and detergent industries. Amylases can be used to form a variety of starch-hydrolyzed products with numerous physical and chemical qualities for these industries (Ozdemir et al., 2011). In these industries, until the 19th century, starch saccharification was achieved by acid hydrolysis using dilute HCl because the understanding of the potential advantages of biological catalysts was limited. Because several types of microbiol amylases have since been found and developed, many advantages of enzymatic starch processing have been shown over chemical starch hydrolysis. These examples include the following: (1) enzymatic processing can be performed at low temperatures compared with the chemical method; (2) it is not necessary to use a corrosion-resistant container for the reaction and the waste water is neutral pH and clean because the hydrolytic reaction of starch by enzymatic processing can be performed at around neutral conditions; (3) glucose yields of enzymatic processing are higher than the chemical method; and (4) chemical methods form unwanted colors and bitter taste compounds in hydrolysates. Therefore, today, starch saccharification achieved using an entirely enzyme-based process (Jansen and Olsen 1999; Sharma and Satyanarayana, 2013).

3. Microbial α-amylases

Microbial amylases are rich in diversity, and several amylases with various properties have already been found and developed (Pandey et al., 2000). Therefore, microbial amylases have high advantages to apply each industry compared with amylases from other sources because each industry requires specific amylases that have the suitable abilities for each application method. Microbial amylases are currently the main starch hydrolyzing enzymes that are used for starch saccharification and other applications, except for β-amylase from malt. However, among these industrial amylases, microbiol α-amylase is one of the most in-demand industrial enzymes in several industries.

In this section, we summarize the types of microorganisms that are sources of various α-amylases.

3.1 Fungal α-amylases

In the Asian region, filamentous fungal α-amylases have been used traditionally for making fermentation foods because they are suitable for solid-state fermentation (Rahardjo et al., 2005). Especially in Japan, genus Aspergillus, which are called koji molds, have been used as a providers of various secretory enzymes, i.e., lipases, proteases, and amylases, in making traditional fermentation foods, such as sake, shoyu (soy sauce), miso (soy-bean paste), and koji amazake (Sakaguchi et al., 1992; Machida et al., 2008; Kitamoto 2015; Ichishima, 2016; Oguro et al., 2019).
For years, fungal α-amylases also have been used in the baking industry. α-Amylase is added to the dough during the bread baking process. The amylases degrade the damaged starch in wheat flour into small dextrins, which allows the yeast to work continuously during dough fermentation, proofing, and in the early stage of baking, resulting in improved bread volume and crumb texture. In addition, the small oligosaccharides and sugars such as glucose and maltose that are produced by α-amylases that enhance the Maillard reactions that are responsible for browning of the crust and the development of an attractive baked flavor (Synowiecki, 2007; Goesaert et al., 2009).

In 1894, Dr. Jokichi Takamine commercialized taka-diastase (amylase), which was produced by the koji mold *Aspergillus oryzae*, and since then, many kinds of α-amylases from filamentous fungi, genera *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, and other fungi have been used for several industrial applications (Pandey et al., 2000). Moreover, it was reported that the thermophilic fungus *Thermomyces lanuginosus* is an excellent producer of amylase (Mishra and Maheshwari, 1996; Rubinder et al., 2002; Kunamneni et al., 2005).

Basidiomycete yeast strains belonging to genus *Saitozyma* (formerly genus *Cryptococcus*) also produce raw starch-degrading α-amylases (Iefuji et al., 1996; Wanderley et al., 2004; de Barros et al., 2009; Galdino et al., 2008; Galdino et al., 2011).

### 3.2 Bacterial α-amylases

Many different types of bacterial α-amylase have been developed from several microorganisms so far, so that the appropriate bacterial α-amylase can be selected from the bacterial α-amylase library. Among these bacterial α-amylases, the enzymes from the genus *Bacillus*, i.e., *B. subtilis*, *B. megaterium*, *B. licheniformis*, *B. stearothermophilus*, and *B. amyloliquefaciens*, have high potential for application in several industrial processes such as in the food, fermentation, textiles, and paper industries (Pandey et al., 2000; Jujjavarapu and Dhagat, 2019).

However, the saccharification process is performed at a relatively high temperature to avoid contamination by various bacteria. In this case, thermostable α-amylases are required for the saccharification process. The thermophilic α-amylases are also found from several extreme thermophilic bacteria, such as thermophilic archaea, actinomycete, and other bacteria strains (Pandey et al., 2000; Bruins et al., 2001; Bertoldo and Antranikian, 2002). The thermophilic α-amylases are summarized in detail in the sections below.

### 4. Bacterial α-amylases in the extremozymes

Microorganisms have established a diversity of molecular strategies to adapt to habiting environments. Extremophilic microorganisms, in particular, have evolved by adapting metabolic enzymes to extreme environments; extremozymes produced by extremophiles possess high catalytic abilities under respective extreme conditions.

In this section, we summarize thermophilic, psychrophilic, acidophilic, and alkaliophilic α-amylases from microorganisms.

#### 4.1 Thermophilic α-amylases

In a typical chemical reaction, increasing the temperature leads to an increase of reaction activity for reaction kinetics. For a reaction that is catalyzed by enzymes, however, increasing the temperature also accelerates the denaturation of enzymes during the reaction step. Thermophilic and thermostable enzymes have been investigated for some industrial reaction steps that use enzymes as catalysts. There is a high demand for thermophilic and thermostable enzymes in the starch-related industries; in particular, thermophilic amylases are essential and used in countless industrial applications, such as in the detergent, textile, brewing and baking, sugar, and paper industries.
(Haki and Rakshit, 2003). In particular, because enzymatic liquefaction and saccharification of starch are performed at high temperatures (100–110°C), thermostable amylolytic enzymes have been currently analyzed to improve the industrial processes of starch degradation.

Thus, several thermophilic amylases have been isolated and developed. In particular, thermophilic α-amylases are also found in several extreme thermophilic bacteria, such as thermophilic archaea, actinomycete, bacteria, and fungi strains, and their enzymatic properties have been reported (Pandey et al., 2000; Bruins et al., 2001; Bertoldo and Antranikian, 2002).

For fungal α-amylases, the optimum temperature for α-amylase from the koji mold A. oryzae is 50 to 55ºC (Kundu and Das, 1970), but α-amylase from the thermophilic fungus Thermomyces lanuginosus or Myceliophthora thermophila has a higher optimum temperature (60ºC) (Jensen and Olsen, 1991; Jensen and Olsen, 1999; Ramkrishna et al.,1993). As described above, because the fungal α-amylases have a relatively low optimum temperature, they may be not suitable for reaction processes that occur at high temperatures.

Bacterial α-amylases have higher optimum temperatures compared with fungal α-amylases. Several hyper thermophilic archaea, which are able to grow at around the boiling point of water, have been isolated. The thermophilic α-amylases from the hyper thermophilic archaea strains may have a potential use in harsh industrial conditions. For example, strains belonging to genus Pyrococcus and Thermococcus produce thermophilic α-amylases that have optimum temperatures of 80–100ºC (Haki and Rakshit, 2003).

Even the mesophilic bacteria strains in genus Bacillus produce thermophilic α-amylases (Goyal et al., 2005; Hmidet et al., 2010); α-amylases from B. licheniformis strains, in particular, are one of the major thermophilic enzymes with an optimum temperature of 90–100ºC (Viara et al., 1993; Hmidet et al., 2008; Bozic et al., 2011). The α-amylase from B. licheniformis has provided an attractive model for investigating the structural basis of thermostability for enzymes, and its 3D structure, along with that of other thermophilic α-amylases, was already known (Machius et al., 1998; Mehta and Satyanarayana, 2016). Moreover, it was suggested that the factors that are responsible for the remarkable thermostability of α-amylase may increase ionic interactions, reduce surface area, and increase packing interactions in the interior (Hwang et al., 1997; Hiteshi and Gupta 2014).

4.2 Psychrophilic α-amylases

The intrinsic characteristics of cold-adapted enzymes (psychrophilic enzymes), which have high activity at low temperatures and thermostability, are extremely valuable for several industrial applications for detergents and for food and beverage preparation (Marx et al., 2007; Cavicchioli et al., 2011; Feller, 2013). The stability and high activity of psychrophilic enzymes at low temperatures are very important properties for use in the industries for the following reasons: (1) enzymatic processing at low temperatures can avoid degradation of the quality for processed foods during processing step; (2) it reduces the growth of other contaminating bacteria during processing step; and (3) it also reduces undesirable chemical reactions that occur at high temperatures (Kuddus et al., 2011). Novel cold-adapted enzymes have recently been discovered and substantially developed, and almost all psychrophilic enzymes are isolated from psychrophiles, which grow optimally at less than 15°C (upper limit of 20°C) (Morita, 1975).

Psychrophilic α-amylases are also very useful in the detergent and food industries. A psychrophilic α-amylase from B. cereus strain GA6 is stable and active at low temperatures (4–37°C), with an optimum temperature of 22°C (Roohi et al., 2013). α-Amylase also is stable and active under alkaline condition at pH 7–11, and it has high detergent-stability (Roohi et al., 2013). The recombinant α-amylase from the marine bacterium Zunongwangia profunda has a high activity at a wide range of temperatures, from 0 to 35°C, and maintains 39 and 46% of activity at 0 and 5°C, respectively (Qin et al., 2014). However, a psychrophilic α-amylase from the Antarctic psychrophile,
Alteromonas haloplanctis, also has been characterized in detail. The \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) values of the \( \alpha \)-amylase are larger than the values determined for the porcine enzyme over a temperature range of 0°C to at least 25°C (Feller et al., 1994). It is the first psychrophilic \( \alpha \)-amylase that has been successfully crystallized and the 3D structure resolved at 1.85 Å (Aghajari et al., 1996). From identification of the structure, they proposed that determining factors of the conformational flexibility, which allows efficient enzyme catalysis in cold conditions, are (1) an increased resilience of the molecular surface, and (2) a less rigid protein core, with less interdomain interactions (Aghajari et al., 1998).

After these reports, the 3D homology models of some psychrophilic \( \alpha \)-amylases have been shown (Ramli et al., 2013; Yang et al., 2017).

### 4.3 Acidophilic \( \alpha \)-amylases

Acidophiles are also known to produce acidophilic \( \alpha \)-amylases. The acidophiles are classified as organisms that can withstand and even thrive in acidic environments having pH values in the range of 1.0 to 5.0, and they have a pH optimum for growth that is less than pH 3 (Baker-Austin and Dopson, 2007). The acidophiles are found in eukaryotes (fungi) as well as prokaryotes (bacteria and archaea), which thrive in a variety of acidic environments, including sulfuric pools and geysers, areas polluted by acid mine drainage, and even in our intestine (Baker-Austin and Dopson 2007; Sharma et al., 2012).

\( \alpha \)-Amylases from acidophilic strains belong to genus Alicyclobacillus have thermostability and acidostability with optimum temperature, 75°C, and optimum pH, 3.0–4.2 (Matzke et al., 1997; Bai et al., 2012). An \( \alpha \)-amylase from the acidophilic bacterium Bacillus sp. DR90 was active in a wide range of pH and temperature having optimal activity at pH 4.0 and 75°C (Asoodeh et al., 2014). In archaea, thermo-acidophilic \( \alpha \)-amylase isolated from Pyrococcus furiosus was optimally active at 100°C and pH 5.5–6.0 (Laderman et al., 1993).

Despite extensive research on acidophiles, very few have been exploited for commercial purposes (Parashar and Satyanarayana 2018).

### 4.4 Alkaliphilic \( \alpha \)-amylases

Alkaliphilic enzymes are produced by alkaliphiles, which grow optimally at pH values above 9, often between 10 and 12, but are unable to grow or grow slowly at the near neutral pH value of 6.5 (Horikoshi, 1999). Alkaliphilic enzymes are widely used for several industrial applications such as leather tanning, paper-pulp bleaching, production of cyclomaltodextrins (CDs), treatment of agricultural and food processing wastes (Horikoshi, 1999; Fujinami and Fujisawa, 2010). Among them, alkaliphilic \( \alpha \)-amylases are very useful in detergent and food industries, as well as psychrophilic \( \alpha \)-amylases (Horikoshi, 1999; Fujinami and Fujisawa, 2010).

In enzymatic detergents, in particular, \( \alpha \)-amylases play a vital role and 90% of all liquid detergents contain the enzymes (Gupta et al., 2003). In the detergent industry, alkaliphilic properties of \( \alpha \)-amylase contributes to its extensive use in detergents, together with psychrophilic properties (Sundarram and Murthy, 2014). The \( \alpha \)-amylase is used to remove starch to improve detergency of laundry bleach composition and bleaching without color darkening (Borchet et al., 1995; Saini et al., 2017).

In 1971, there was a first report about an alkaliphilic \( \alpha \)-amylase from alkaliphilic Bacillus sp. strain A-40-2 (Horikoshi, 1971). The optimum pH of the \( \alpha \)-amylase is pH 10.0–10.5, and it retains 50% of its activity between pH 9.0–11.5 (Horikoshi, 1971). After this great discovery, many \( \alpha \)-amylases from alkaliphilic Bacillus have been reported (Horikoshi, 1999).

However, CDs are synthesized from starch, and its reaction is catalyzed by glucanotransferase (CGTase: EC 2.4.1.19), which catalyzes cyclizing of 1,4-\( \alpha \)-D-glucan molecule through the formation of a 1,4-\( \alpha \)-D-glucosidic bond.
Qi and Zimmermann, 2005. CDs are used in several industries such as industries related to food, pharmaceuticals, cosmetics, chemicals, and agriculture (Del Valle 2004; Szente and Szejtli 2004). CGTase is a member of the α-amylase superfamily, which catalyzes the cleavage of the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Qi and Zimmermann, 2005). Many alkalophilic Bacillus strains produce CGTases and their CGTases are applied to production of CDs (Qi and Zimmermann, 2005).

5. Conclusion

As shown in this review, amylases are vital enzymes that are used in the starch processing industry in the production of starch hydrolysates. Amylases are still gaining importance in many industrial fields, such as clinical, medicinal, analytical chemistry, fine chemical, and pharmaceutical industries, and the demand for the enzymes is expected to increase in the future.

Moreover, applications of amylases are increasing, and according to needs for each industry, novel types of amylases will be developed. Development of novel types of amylases can promote its application in new industrial fields. Therefore, it is reasonable to expect that microbial amylases will play more significant and diverse roles in our life, and further progression of research in screening of novel types of amylases included in "extremozymes" and their applications as industrial enzymes is expected in the future.

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