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Optimization of physico-chemical condition for improved production of hyperthermostable β amylase from *Bacillus subtilis* DJ5

Abhijit Poddar, Ratan Gachhui, Subhas Chandra Jana*

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Abstract

Bacillus subtilis DJ5 was found to produce hyperthermostable β amylase in a complex medium during submerged fermentation. Several physico-chemical parameters affecting microbial growth and enzyme production were optimized. Initial medium pH of 7 and cultivation temperature of 37 °C were found optimal for enzyme production. Among different nitrogen and carbon sources tested for designing an improved media, 0.05% tryptone and 5% starch respectively were most effective for enzyme yield. Little supplementation of lysine (0.03%) remarkably increased enzyme titer (14.24 U/ml). Nitrate (0.03%) played a major role in microbial growth and subsequent enzyme production. Enzyme production was increased upto 1.67 fold in optimized medium. Optimized media resulted in higher enzyme titer (18.32 U/mg) than basal media (10.97U/mg).

Keywords: Hyperthermostable β amylase; *Bacillus subtilis* DJ5; media optimization.

Introduction

Economization of industrial production of enzymes depend on several factors, such as the composition of the culture medium, carbon and nitrogen source, mineral salts, trace elements, type of strain, and fermentation conditions (pH, temperature, oxygen concentration, agitation) (Weuster-Botz 2000; Pinto et al. 2002; Duta et al. 2004). Since 30 to 40% of the production cost of industrial enzymes is estimated to be accounted for by the cost of the growth medium (Joo et al. 2002), designing a fermentation medium is of critical importance because medium compositions can significantly affect product concentration, yield and volumetric productivity. Designing the medium is a laborious, expensive, open-ended, often time-consuming process involving many experiments. The reach of optimized fermentation conditions, particularly associated to physical and chemical parameters, is of primary and great importance for the development of any process, due to their impact upon its economy and practicability.

Several different approach of media optimization investigated (Kennedy and Krouse 1999), one factor at a time optimization method has found tremendous popularity and wide application (Amrane and Prigent 1993; Bajpai et al. 1992; Houng et al. 1989). The rationale behind the one-at-a-time strategy is to keep the concentration of all medium components constant except one. The concentration of this medium component is then changed over a desired range. This strategy has the advantage that it is simple and easy. Most significantly, the individual effects of medium components can be seen on a graph, without the need to revert to statistical analysis.

Thermostable amylolytic enzymes has been widely used in starch processing, brewing and sugar production (Leveque et al. 2000) desizing in textile industries (Hendriksen et al. 1999) and in detergent manufacturing processes (Lin et al. 1998). Thermostability offers enzymatic stability during high temperature of starch gelatinization (100-110 °C) and liquefaction (80-90 °C) that is prerequisite for starch processing (Haki and Rakshit 2003). It also reduces cooling costs, allows better solubility of substrates and reduced risk of microbial contamination (Turner et al. 2007).

Bacillus subtilis DJ5 has been found to produce hyperthermostable β amylase (Poddar et al. 2011a). The enzyme was fully stable at 121 °C for 15 min. Previously Shen et al. reported one thermosable β amylase that was active at 80 °C (Shen et al. 1988). In that respect this enzyme has a leading edge over all previously published enzymes and has illuminated opportunity of using this enzyme in industrial applications. Moreover, we have successfully immobilized the enzyme in gelatin matrix (Poddar and Jana 2011) and evaluated its efficacy toward processing of raw agricultural product (Poddar et al. 2011b).

In this study, with a view of using this enzyme in industry, production media of *Bacillus subtilis* DJ5 has been optimized for

Abhijit Poddar, Subhas Chandra Jana*

Post Graduate Department of Microbiology, Bidhannagar College (Govt. of West Bengal), West Bengal, India

*Email: subhasjana1959@gmail.com

Ratan Gachhui

Department of Life Science & Biotechnology, Jadavpur University, West Bengal, India

improved production of hyperthermostable β amylase. Moreover physical parameters for growth and optimum enzyme production have also been optimized. As amylase occupies 25% of the enzyme market (Burhan et al. 2003), this work will illuminate requirements of this strain for maximum exploitation of its amylase producing machinery.

Materials and methods

Working strain

Bacillus subtilis DJ5 (GenBank accession number GU357825) (Poddar et al. 2011) was used in this study. This strain was isolated and subsequently mutated by physical and chemical mutagenesis in our laboratory.

Cultivating media

Culture was transferred aseptically in 250 mL conical flask containing 100 mL sterile Starch Peptone medium (pH 7) containing (gm/L): Peptone, 0.9; $(NH_4)_2HPO_4$, 0.4; KCl, 0.1; $MgSO_4,7H_2O$, 0.1; NaH_2PO_4, $2H_2O$, 0.5; soluble starch (Sigma, USA), 5. The flask was kept in BOD shaker incubator at 160 rpm at 37°C for 6.5 hour.

Optimization of physical parameters for growth and β amylase production

Microbial growth (OD_{660}) and β amylase production were tested at different temperatures after incubating inoculated broth medium in BOD shaker incubator in shaking condition (160 rpm) for 6.5 hours. Similarly, media pH was also fluctuated with addition of 0.1N HCl and/or 0.1N NaOH and was sterilized after autoclaving. Media of different pH was separately inoculated with Bacillus subtilis DJ5 and allowed to ferment for 6.5 hour in rotary shaker incubator at 37 °C to determine optimum pH for microbial growth and beta amylase production.

Optimization of media components for beta amylase production

Several complex nitrogen sources (protease peptone, soya peptone, yeast extract, beef extract, tryptone), amino acid sources (Trptophan, Thymine, Arginine, Glycine, Lysine, Leucine, Methionine, Valine), metal ions source (salt sources like Na_2CO_3, $NaHCO_3$, $NaNO_3$, Na_2SO_4, NaCl, $NaNO_2$, KI, K acetate, K-tartarate, K_2SO_4, $NaMoO_4$) and carbohydrate sources were varied in different concentrations in basal medium one at a time. All other medium ingredients in basal medium were kept constant when one component was varied. Optimum concentration of ingredient was selected by determining β amylase activity of cell free extract of six and half hour fermented broth.

Assay of β amylolytic activity

β amylolytic activity was measured by the method of Bernfeld (1955). Assay mixture contains 0.5 mL of 0.1M phosphate buffer (pH 6.9), 1 mL soluble starch (0.5% w/v, Sigma Chemicals) and 0.1 mL of crude enzyme. Control was prepared as same without adding substrate. The reaction mixture was incubated at 100°C for 15 min. Enzyme-substrate reaction was then stopped by addition of 1 mL 2M NaOH. Both the assay mixture and control were then allowed to boil in boiling water bath for 10 min after addition of 0.5 mL of 3, 5-dinitrosalisylic acid reagent (Merck, Germany). After cooling the assay mixture at room temperature, absorbance were measured spectrophotometrically (Elico, India) at 540 nm. Amount of maltose released was measured from standard curve of maltose. One unit (U) of β amylolytic activity was defined as the amount of enzyme releasing 1 µmol of maltose equivalent per minute per ml from soluble starch (Sigma) under the standard assay conditions. The specific activity of the enzyme (U/mg of protein) was also determined by measuring protein content of enzyme using bovine serum albumin (BSA) as the standard, according to Lowry et al. (1951). All the experiments were performed in triplicates. The relative β amylase activity was defined as percentage of maximum specific activity measured in assay.

Cell Growth and Cell Leakage

Cell growth in freely suspended cultures were determined spectrophotometrically (Elico, India) by measuring the optical density at 660 nm.

Results

Optimization of physical conditions

a) Temperature optima for growth and beta amylase production

Bacillus subtilis DJ5 showed true mesophilic character. Growth is optimum at 37 °C and is almost inhibited at 45 °C (Fig. 1). Higher metabolic activity at 37°C also resulted in maximum production of β amylase evidenced from highest enzyme titer (10.97U/mg).

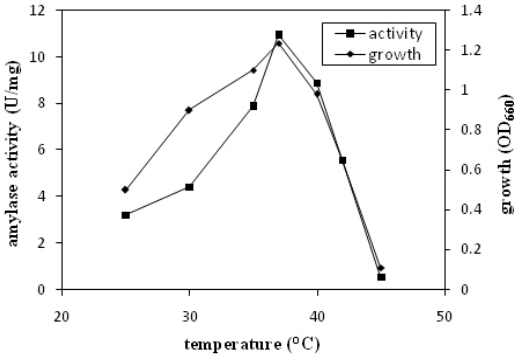

Fig. 1: Effect of temperature on growth and β amylase production.

b) Effect of pH on growth and beta amylase production

Organism's growth was found to be pH dependent behaving like neutrophiles. Growths at 37 °C in different pH medium showed this organism grow best at pH 7 (OD_{660} = 1.2) under aerobic condition (Fig. 2). Higher or lower pH significantly reduced both growth and enzymatic activity.

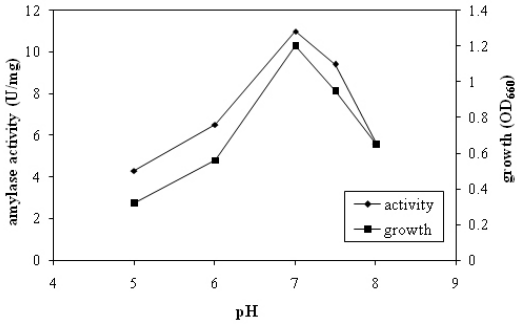

Fig. 2: Effect of pH on growth and β amylase production.

Formulation of fermentation medium

a) Effect of nitrogen source

Several organic nitrogen sources tested, basal media supplemented with 0.05% tryptone showed maximum production of enzyme (6.84 U/ml) followed by soya peptone (6.19 U/ml) and protease peptone (5.94 U/ml) (Fig. 3). Yeast extract also gave higher enzyme productivity (6.23 U/ml) but at a higher concentration i.e. 0.09%. Beef extract probably showed negative impact on β amylase activity as it gave very poor enzyme titer (3.18 U/ml at 0.15% beef extract concentration). Higher protein content in fermentation media showed overall a reduction in enzyme synthesis.

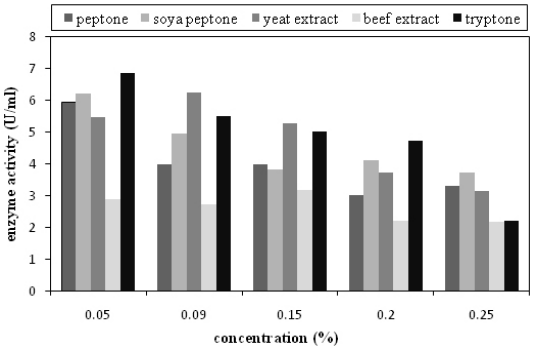

Fig. 3: Effect of nitrogen source on β amylase production. Process condition: Medium used - basal media (pH 7) without any nitrogen source, temperature- 37 °C.

b) Effect of various amino acids on β amylase activity

Though basal media do not contained any types of external free amino acid sources, addition of free amino acids for *Bacillus subtilis* DJ5 growth medium remarkably affected enzyme production (Fig. 4). Appreciable stimulation in enzyme titer was recorded for 0.03% supplementation of lysine (14.24 U/ml), valine (13.37 U/ml), methionine (8.68 U/ml), glycine (7.68 U/ml) andleucine (7.33 U/ml), Arginine at 0.09% concentration stimulated enzyme synthesis a little (6.44 U/ml). Tryptophan and thymine acted antagonistly reducing enzyme titer to a higher extent.

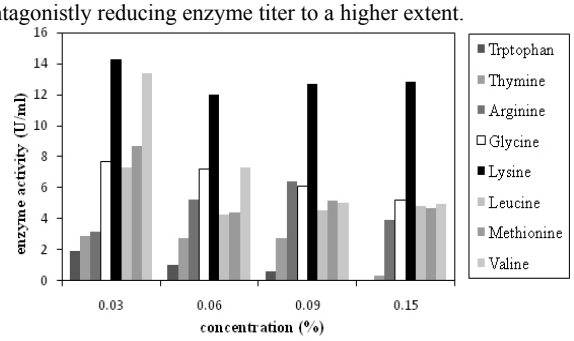

Fig. 4: Effect of amino acid on β amylase production. Process standard: Media used-basal media (pH 7) + amino acid source, temperature- 37 °C.

c) Effect of different metal salts on β amylase activity

Among the four different metal salts (gm/L) ($(NH_4)_2HPO_4$, 0.4; KCl, 0.1; $MgSO_4,7H_2O$, 0.1; NaH_2PO_4, $2H_2O$, 0.5) present in the basal medium, one at a time deletion of KCl and $(NH_4)_2HPO_4$ showed no decrease in rates of enzyme synthesis but $MgSO_4,7H_2O$ and NaH_2PO_4, $2H_2O$ were essential for enzyme production. So during variation of different metal salts in fermentation media concentration of $MgSO_4$, $7H_2O$ and NaH_2PO_4, $2H_2O$ were remained undisturbed.

Except bicarbonate and carbonate salts, all metal ions tested in growth medium for stimulation of enzyme production showed nearly similar stimulatory effect (Fig. 5). Among them, nitrate as sole salt source resulted in maximum production of enzyme (7.7 U/mg) at very low concentration (0.03%). Bicarbonata and carbonate salts at any concentration impacted negatively reducing enzyme synthesis at a basal level.

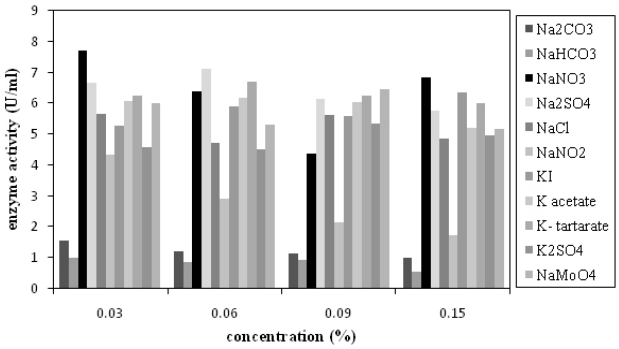

Fig. 5: Effect of metal ions on β amylase production. Process standard: Media used-basal media (pH 7) without any salt source, temperature- 37 °C.

d) Effect of starch concentration on growth and β-amylase activity

As starch is primarily considered as principle substrate for amylases, different concentrations of starch (Sigma, USA) were varied in growth medium to determine best substrate concentration for microbial growth and enzyme activity (Fig. 6). Result showed that starch at a concentration of 5mg/ml (5% v/v) is best for the organisms' growth. Even at this concentration *Bacillus subtilis* DJ5 was able to exploit its amylase produce machinery at highest efficiency as indicated by maximum enzyme activity (10.97 U/mg). Though microbial growth was not affected by higher concentration of substrate in fermentation media, yet β amylolytic activity was found to reduce steadily with the increase of starch concentration in fermentation media.

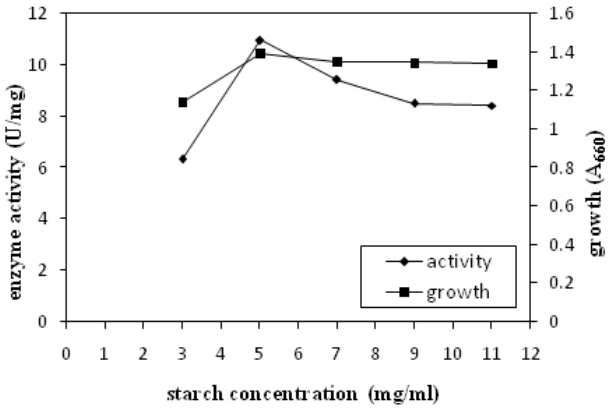

Fig. 6: Effect of starch concentration on growth and β amylase production. Process standard: Media used-basal media (pH 7) without starch sources + different starch concentration, temperature- 37 °C.

e) Effect of different carbon sources on growth and β-amylase activity

Different carbon sources, monosaccharides (glucose, fructose, galactose, xylose, arabinose), disaccharides (maltose, sucrose, melibiose, trehalose), trisaccharide (rafinose) and polysaccharide (starch, cellulose) and sugar alcohol (mannitol, inositol) were tested

separately at a same concentration (5mg/ml) in the fermentation media to determine their effect on β amylase production (Table 1).

Though monosaccharides are not substrate for amylases, yet production medium containing galactose and xylose somehow was able to produce higher amount of β amylase 14.78 U/mg and 12.37 U/mg respectively without affecting microbial growth. Aldopentose (arabinose) and aldohexose (glucose) and ketohexose (fructose) remarkably stimulated microbial growth but failed to stimulate enzyme production. Moreover their presence in fermentation media showed some adverse effect on β amylase production as evidenced from lower enzyme titer.

Non reducing disaccharide trehalose completely abolished production of β amylase (0.634 U/mg) though it supported microbial growth. Maltose with α (1-4) bond and melibiose with α (1-6) linkages showed no appreciable stimulation or inhibition on rates of β amylase synthesis. Interesting to note that though maltose is the product of starch hydrolysis by β amylase, yet presence of maltose in fermentation media as sole carbon source did not affected enzyme production indicating a constitutive nature of enzyme synthesis by *Bacillus subtilis* DJ5.

As β(1-4) linkages are not susceptible to β amylase attack, polysaccharide cellulose failed to secret β amylase in extracellular media (0.545 U/mg). Similarly, sugar alcohols also showed adverse effect on β amylase.

Table 1: Effect of carbon sources on growth and β amylase production. Process standard: Media used-basal media (pH 7) without carbon sources, temperature- 37 °C.

Carbohydrate (0.5%)	Growth		pH		Sp. Activity (U/mg)
	Initial	Final	Initial	Final	
Glucose	0.161	1.702	6.73	5.22	4.972
Fructose	0.122	1.456	6.09	4.80	4.07
Galactose	0.109	1.163	6.7	7.01	14.78
Xylose	0.111	1.153	6.23	6.76	12.37
Arabinose	0.106	1.580	5.86	4.56	6.90
Maltose	0.104	1.542	6.76	5.54	10.80
Sucrose	0.107	0.958	5.67	4.60	4.58
Melibiose	0.130	1.533	5.84	5.84	9.94
Treahalose	0.124	1.314	6.50	5.30	0.634
Rafinose	0.119	1.568	6.78	5.65	6.96
Mannitol	0.121	1.280	6.88	4.85	1.026
Inositol	0.141	0.950	6.79	4.77	0.534
Starch	0.158	1.396	6.67	6.34	10.97
cellulose	0.121	1.134	6.50	6.73	0.545

Comparative growth and β amylase production from Bacillus subtilis DJ5 in basal and optimized media

Variation of each components of basal media in one at a time approach indicated that few components of basal media needs some higher concentration, few are not required at all and few must be incorporated to produce an optimized media that will not only support microbial growth but will allow higher production of enzyme. In that view, a new optimized media (pH 7) was formulated with following composition (gram per liter): tryptone, 0.5; $(NH_4)_2HPO_4$, 0.4; $MgSO_4,7H_2O$, 0.1; NaH_2PO_4, $2H_2O$, 0.5; $NaNO_3$, 0.3; lysine, 0.3; galactose, 0.5; soluble starch (Sigma, USA), 5. Media inoculated with 6% inoculum was allowed to ferment for 6.5 hours in a rotary shaker incubator at 37°C. Microbial growth and enzyme production was compared with that of basal media. Result indicated that both basal and optimized media allowed slightly higher microbial growth. Enzyme production was increased upto 1.67 fold in optimized medium (Fig. 7). Optimized media resulted in higher enzyme titer (18.32 U/mg) than basal media (10.97U/mg).

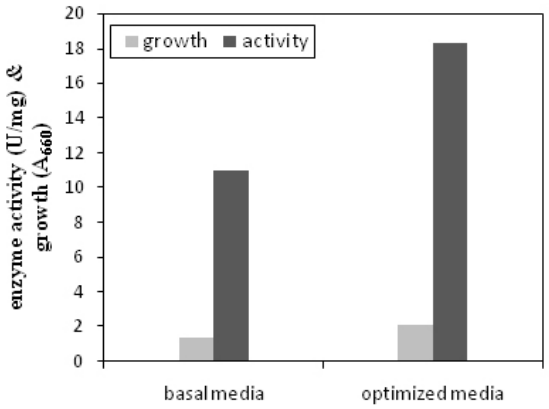

Fig. 7: Comparison of growth and β amylase production in basal and optimized medium.

Discussion

Media is said to be optimized or improved if it show enhanced performance regarding production of industrially important enzymes, or any other economically viable compound. During industrial processing of starch, solid starches are enzymatically hydrolyzed at elevated temperature (90-110 °C) (Leveque et al. 2000). So β amylase must be thermostable at or above 100 °C. Till date no such thermostable β amylase has been isolated (Shen et al. 1988). In that respect β amylase from mutated strain of *Bacillus subtilis* DJ5 showed 100% catalytic stability at 121 °C for 15 min (Poddar et al. 2011a). In order to be applied in industry, optimization of fermentation parameters was necessary.

Regarding optimal growth and pH requirement, this organism has showed true mesophilc and neutrophilic character showing maximum growth at 37 °C and pH 7 respectively. Similar findings were suggested by Ray et al. 1995. Such growth requirements will make it easier to maintain and preserve the organism providing easier alternative of culture handling.

Borrowing media from previously published work has been considered a good alternative of media optimization to start with as it is already been optimized for a particular organism for production of industrially important products (Kennedy and Krouse 1999). Ray et al. 1995 has previously used starch peptone media (pH 7) for improved production of β amylase from *Bacillus megaterium* B6. This media was considered as basal media in this work as this study also aimed in improved production of β amylase from same genus of bacterium i.e. *Bacillus*.

Bacillus subtilis DJ5 has shown to utilize casein enzyme hydrolysate (tryptone) at a very low concentration of 0.05% more efficiently than any other nitrogen sources. This may be due to less complexity was preferred for microbial growth and enzyme production. This explanation can also be applied generally as more complex animal protein sources (peptone, beef extract) was less stimulant of microbial growth than less complex plant sources (soya peptone).

Supplementation of small amounts of free amino acids has been found beneficial for higher production of enzyme. Though organism was not an auxotrophic mutant and can synthesize its amino acids of its requirements, presence of lysine (0.03%) positively stimulated its amylase producing machinery commanding an increased production of enzyme. Alternatively it can be said that the enzyme itself contains greater amount of lysine residues in its structural framework so presence of lysine in growth media has made it easier

to incorporate those residues in synthesis of enzyme. As structural elucidation of β amylase was outside the scope of this study, determination of lysine contents in beta amylase was not possible.

Present study indicated that hyperthermostable β amylase production is highly dependent on two metal salts, $MgSO_4,7H_2O$ and NaH_2PO_4, $2H_2O$. In this respect this organism has similarities in growth and amylase production requirements with different published reports (Ray et al. 1995; Bajpai et al. 1992; Asgher et. al. 2007). But interestingly there was no reports that nitrate salts can stimulate enzyme production. Result of this study indicated $NaNO_3$ at 0.03% concentration stimulated growth and enzyme production to an appreciable extent (7.7 U/mg).

Greater profit in industrial production is partly related with the lesser overall cost of production media. To lower down media budget, continuous efforts has been given to use low cost carbon sources (Kadam and Newman 1997) minimizing their concentration to a level where growth and production is not compromised. For maximum production of β amylase this organism needs only 0.5% starch concentration. Such lower concentration of substrate requirement has been previously reported by Ray et al. 1955 and will be beneficial from industrial point of view.

Ability of this organism to produce higher amount of β amylase irrespective of presence of starch in fermentation media indicates constitutive nature of the enzyme as opposed by many studies where presence of glucose, maltose significantly reduced beta amylase activity (Ray et al. 1995).

Moreover providing both starches and monosaccharide galactose (0.5%) in fermentation can stimulate synthesis of β amylase upto 1.67 fold in optimized media. Readily available supply of monosaccharide may encourage the organism to grow without stress and as a result increase the constitutive yield of β amylase.

Conclusion

Industrial production of hyperthermostable β amylase from *Bacillus subtilis* DJ5 is possible by growing the culture at 37 °C in an optimized media (pH 7) containing (gram per liter): tryptone, 0.5; $(NH_4)_2HPO_4$, 0.4; $MgSO_4,7H_2O$, 0.1; NaH_2PO_4, $2H_2O$, 0.5; $NaNO_3$, 0.3; lysine, 0.3; galactose, 0.5; soluble starch (Sigma, USA), 5. The optimized media affect microbial growth (OD_{660} = 2.11) to a very lesser extent compared to growth in basal media (OD_{660} = 1.39) but interestingly enhance enzyme production upto 1.67 fold. This media can be termed as 'production committed' rather than growth committed.

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