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Simultaneous production and decomposition of clavulanic acid during *Streptomyces clavuligerus* cultivations

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Abstract Clavulanic acid (CA) was produced by *Streptomyces clavuligerus* in medium containing glycerol and soy meal or soy meal extract. With regard to growth and CA productivity, the microorganism showed significant differences if solid soy meal as such or its extract were applied as the major nitrogen source. If the extract is used, growth and CA production take place simultaneously and in the stationary phase the CA concentration is stagnant or reduces. If soy meal is used, growth is threefold faster and CA is only generated in the stationary phase. In the case of using the soy meal extract, the decrease of the CA concentration is mainly due to decomposition or re-metabolisation of CA in the presence of the microorganism. This conclusion is supported by in vivo and in vitro data on CA decomposition.

Introduction

Clavulanic acid (CA) is a potent β-lactamase inhibitor produced by *Streptomyces clavuligerus* simultaneously with other β-lactams such as cephamycin C, penicillin N, deacetoxycephalosporin C and other metabolic intermediates of the two biosynthetic pathways involved (Reading and Cole 1977; Brown et al. 1984). Nowadays, the combination of CA with amoxicillin is the most successful example of the use of traditional β-lactam antibiotics sensitive to β-lactamases together with β-lactamase-inhibiting substances.

The biosynthesis of CA is still not completely clarified. Many intermediates and enzymes involved in the pathway are now known (Salowe et al. 1991; Valentine et al. 1993) and some work has been done on the regulation and optimisation of CA formation (Romero et al. 1984; Lebrihi et al. 1987), although the major part of previous work refers mainly to the production of cephamycin C. As for the most secondary metabolites, production levels of CA are dependent on several regulating factors in addition to typical process parameters such as temperature, pH and partial pressure of O_2, PO_2. Some authors have already investigated the influence of the nature of the carbon and nitrogen sources on the production of cephamycin C by *S. clavuligerus* in defined and complex media (Aharonowitz and Demain 1978; Lebrihi et al. 1988). It has been shown that, under non-limiting fast growth conditions, the beginning of the production phase will be delayed up to the end of the trophophase, whereas tropho- and idiophase are practically simultaneous under slower growth conditions. This is a typical phenomenon for secondary metabolism related to repression or inhibition effects exerted by high concentrations of catabolites on the enzymes responsible for the production.

In this work, the production of CA was studied with the aim of obtaining CA-containing supernatant for further studies of downstream processing. The cultivations were undertaken in complex media containing glycerol and soy meal or soy meal extracts as the main nutrient sources. The analysis of the different growth and production patterns obtained could be partially explained by repression or inhibition effects, but it was concluded that an additional effect influencing the productivity could be the decomposition of CA in the medium under production conditions. This supposition was then further investigated through in vivo and in vitro stability tests for the β-lactamase inhibitor CA.

Materials and methods

Chemicals and medium components

Soy meal was purchased from Raiffeisen Genossenschaft, Braunschweig, Germany, and ground to a particle size fraction smaller

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than 1 mm. Meat peptone, K_2HPO_4 and other reagents were from Merck, Darmstadt, Germany. Augmentan Tabs (the trade name for the therapeutic formulation of CA, consisting of a combination of potassium clavulanate and amoxycillin trihydrate in a 1:4 ratio, 625 mg/tablet, SmithKline Beecham Pharma, München, Germany) were bought and used as standard for the high-pressure liquid chromatography (HPLC) method to quantify CA.

Microorganism

Streptomyces clavuligerus (DSM 738) was delivered as a lyophylised culture from DSM (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). Spores were obtained from sporulation agar (Mendelovitz and Aharonowitz 1982), spore suspensions were prepared as described by Hirsch and Ensign (1976) and maintained in 20% glycerol at −70°C. The viability of the deep-frozen cultures was periodically controlled by plating them onto ISP2 agar (malt extract 10 $g \cdot l^{-1}$, yeast extract 4 $g \cdot l^{-1}$, glucose 4 $g \cdot l^{-1}$, agar 12 $g \cdot l^{-1}$, pH 7.2) and counting the colonies formed.

Cultivation conditions

All cultivations were started from spore suspensions containing 5×10^8 colony formation units per millilitre (cfu $\cdot$ ml^{-1}). The spore reactivation step was performed in baffled 100-ml shaking flasks containing ISP1 medium (yeast extract 3 $g \cdot l^{-1}$, tryptone 5 $g \cdot l^{-1}$, pH 7.0) with an inoculum density of 10^6 cfu $\cdot$ ml^{-1} at 27°C and 120 rpm for 24 h. Aliquots from this liquid culture (5% v/v) were used for inoculating the pre-culture medium (glycerol 20 $g \cdot l^{-1}$, soy meal extract 200 $ml \cdot l^{-1}$, peptone 5 $g \cdot l^{-1}$, pH 7.0), which was incubated in unbaffled 1000-l shaking flasks at 27°C and 120 rpm for 36 h and then used for inoculating the production medium at a 5% v/v ratio. The production step followed in 10-l fermenters (solids-free medium: glycerol 4–12 $g \cdot l^{-1}$, soy meal extract 300 $ml \cdot l^{-1}$, peptone 10 $g \cdot l^{-1}$, phosphate 0.8 $g \cdot l^{-1}$, or solids-containing medium: glycerol 4–12 $g \cdot l^{-1}$, soy meal 30 $g \cdot l^{-1}$, peptone 10 $g \cdot l^{-1}$; pH 6.5, 27°C, and agitation with three six-bladed turbines at 300 rpm, aeration rate 1 vvm) for 70–96 h. The fermenter was continuously fed with a 100 $g \cdot l^{-1}$ glycerol solution at variable flow rates after the glycerol concentration attained values under 4 $g \cdot l^{-1}$ to keep the glycerol concentration in the medium between 1 and 4 $g \cdot l^{-1}$. The soy meal extracts were obtained by autoclaving a suspension of 60 g soy meal per litre of water at 121°C for 1 h and separating the supernatant ($\approx$600 ml) by decantation.

Stability tests

The in vitro stability of CA in cultivation supernatants at different pH values and at 21°C was investigated by centrifuging samples taken during the stationary phase of the cultivation, adding a diluted HCl solution to attain the desired pH value and following the degradation of the product by taking samples at regular time intervals and quantifying the remaining product by HPLC.

Quantification of CA

CA was quantified by HPLC using a reversed-phase column (Lichrosorb C-8 3 µm, 100 × 4 mm) and pre-column derivatisation with imidazole (adapted from Foulstone and Reading 1982). The eluent was composed of KH_2PO_4 50 mM, pH 4.5, buffer:methanol 70:30 and the flow rate was 0.5 $ml \cdot min^{-1}$ (pump Biotronik BT 3020). Samples (600 µl) containing 0–0.2 mM clavulanate (0–40 $mg \cdot l^{-1}$) were derivatised with 150 µl imidazol reagent (10.31 g imidazole analytical grade to 50 ml solution, pH 6.8 with deionised water and 5 M HCl) for 30 min at room temperature and injected through a 20-µl loop. The derivatised product was detected by UV absorbance (Beckman 163 variable wavelength UV detector) at 311 nm. The method was calibrated by using 0.2 mM potassium clavulanate solution made from Augmentan Tabs.

Other analytical methods

Glycerol was quantified with a Boeringer-Mannheim enzymatic test kit. Phosphate was quantified by spectrophotometry after reaction with ammonium heptamolybdate. The cell dry weight (CDW) was determined gravimetrically for the cultivations in solids-free medium, and was estimated from the optical density at 600 nm (OD_{600}) measured for samples decanted for 15 s for the cultivations in medium containing soy meal (CDW $\approx 0.5 \times OD_{600}$).

Results

Fermentation runs

Fed-batch cultivations in the 10-l bioreactor were carried out with different initial concentrations of glycerol in the medium. Additionally, the medium contained either soy meal extract or soy meal as the complex carbon and nitrogen source. Figures 1–4 show a series of runs with low and high initial concentrations of glycerol for both cases. Table 1 gives the calculated average values for the specific growth rate, μ, the specific glycerol consumption rate, σ, and the specific production rate, ν, for the same experiments during the growth and the stationary phases. To estimate the rates in the stationary phase, an average biomass concentration was calculated from the experimental data.

From the results obtained in this set of experiments it could be observed that the concentration of CA during the stationary phase behaved differently in the presence or in the absence of soy bean meal particles in the cultivation medium, pointing sometimes to a decline in the product concentration in the last case. To further investigate this behaviour, additional cultivations were done with medium containing soy bean meal extract and different initial medium compositions. Figure 5 shows the specific CA concentration profiles during the stationary phase for these experiments, where the decline of the product concentration can be clearly observed.

In vitro stability tests

To search for a relation between the apparent decay observed in the product concentration profiles of the cultivations with medium containing soy bean meal extract and the natural decomposition reaction of CA in aqueous buffered solutions, stability tests were carried out with supernatants of samples drawn from the

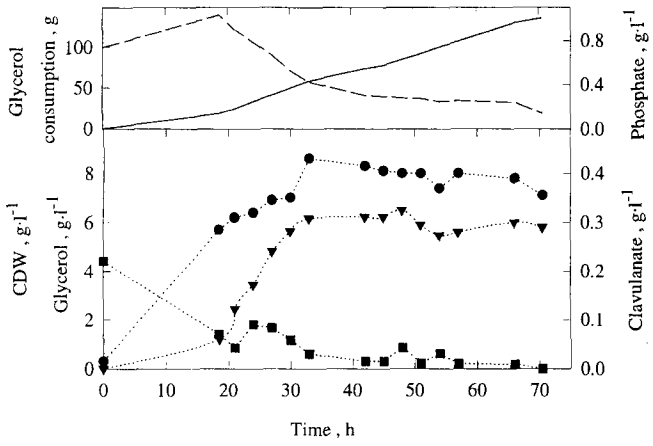

Fig. 1 Time course for changes in biomass (●), glycerol (■), clavulanic acid (▼), total glycerol consumption (—) and phosphate (– –) for a fed-batch cultivation with the following medium: glycerol 4.4 $g \cdot l^{-1}$, soy meal extract 300 $ml \cdot l^{-1}$, meat peptone 10 $g \cdot l^{-1}$, K_2HPO_4 0.8 $g \cdot l^{-1}$; glycerol feeding after 20 process hours. (*CDW* Cell dry weight)

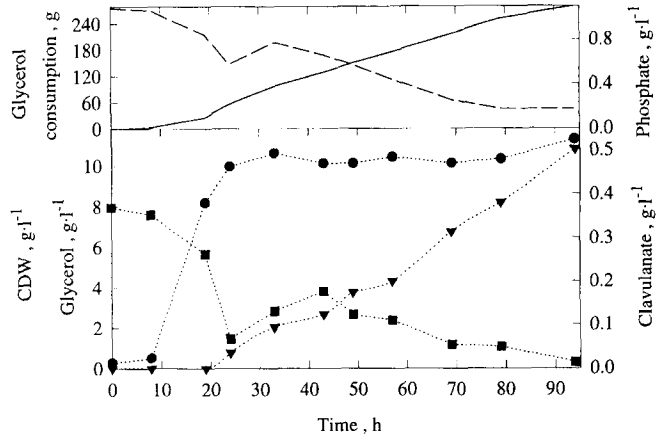

Fig. 3 Time course for biomass (●), glycerol (■), clavulanic acid (▼), total glycerol consumption (—) and phosphate (– –) for a fed-batch cultivation with the following medium: glycerol 7.8 $g \cdot l^{-1}$, soy meal 30 $g \cdot l^{-1}$, meat peptone 10 $g \cdot l^{-1}$; glycerol feeding after 26 process hours

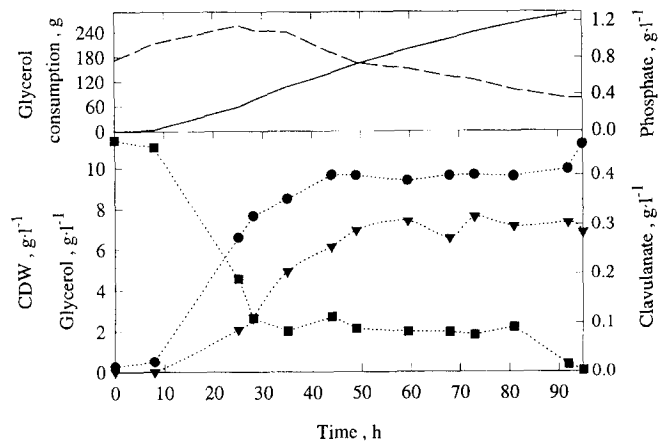

Fig. 2 Time course for biomass (●), glycerol (■), clavulanic acid (▼), total glycerol consumption (—) and phosphate (– –) for a fed-batch cultivation with the following medium: glycerol 11.3 $g \cdot l^{-1}$, soy meal extract 300 $ml \cdot l^{-1}$, meat peptone 10 $g \cdot l^{-1}$, K_2HPO_4 0.8 $g \cdot l^{-1}$; glycerol feeding after 27 process hours

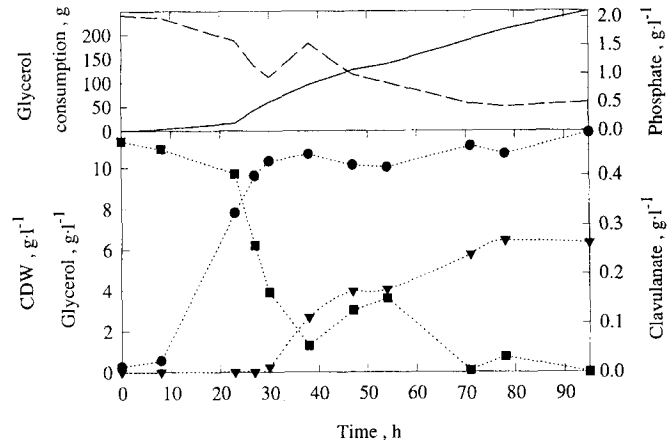

Fig. 4 Time course for biomass (●), glycerol (■), clavulanic acid (▼), total glycerol consumption (—) and phosphate (– –) for a fed-batch cultivation with the following medium: glycerol 11.3 $g \cdot l^{-1}$, soy meal 30 $g \cdot l^{-1}$, meat peptone 10 $g \cdot l^{-1}$; glycerol feeding after 42 process hours

stationary phase, i.e. the in vitro decomposition of CA was studied in samples from cultivations with soy meal extract medium after separating the cells by centrifugation. The measured CA decomposition can be represented by an irreversible first-order reaction with a kinetic constant, k_1, which can be calculated from the slope of the logarithmic decomposition curves. Figure 6 shows the calculated k_1 values in the supernatant samples as a function of the pH. In the same graph are also shown the apparent in vivo decomposition rates estimated from the declining CA concentration profiles in the cultivations with extract medium during the stationary phase, some of which are shown in Fig. 5.

Discussion

When comparing the results presented in Figs. 1–4 some significant differences in the growth and CA production patterns can be discerned. First of all, biomass growth in the medium containing soy meal (Figs. 3, 4) is faster and reaches higher biomass concentrations than in the medium with soy meal extract. Estimations of the specific growth rates indicate that the growth rate is roughly 3 times larger in the presence of soy meal than in the presence of its extract (Table 1). As the extract is obtained from autoclaving a soy meal suspension, one should expect that the initial nutrient

Table 1 Average values for specific growth rate (μ), specific glycerol consumption rate (σ) and specific production rate (ν) during the growth phase and stationary phase for fed-batch cultivations of *S. clavuligerus*

No.[a]	Cultivation Medium	Growth phase			Stationary phase			
		μ (h^{-1})	σ ($g \cdot g^{-1} \cdot h^{-1}$)	ν ($g \cdot g^{-1} \cdot h^{-1}$)	CDW_{avrg} ($g \cdot l^{-1}$)	μ (h^{-1})	σ ($g \cdot g^{-1} \cdot h^{-1}$)	ν ($g \cdot g^{-1} \cdot h^{-1}$)
1	Soy meal extract	0.040	0.066	0.0022	7.9	0.00	0.280	0.00
2	Soy meal extract	0.038	0.071	0.0011	9.7	0.00	0.289	0.00
3	Soy meal	0.111	0.072	0.00	10.4	0.00	0.308	0.0007
4	Soy meal	0.116	0.075	0.00	10.6	0.00	0.262	0.0004

[a] The numbers correspond to Fig. 1–4.
[b] CDW_{avrg} refers to the average cell concentration in the stationary phase

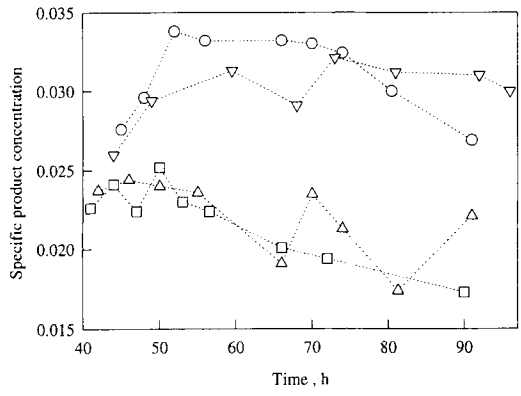

Fig. 5 Specific clavulanic acid concentration profiles during the stationary phase for selected cultivations with soy meal extract medium and different initial glycerol (G) and phosphate (P) concentrations: (○) G = 4.0 $g \cdot l^{-1}$, P = 1.0 $g \cdot l^{-1}$; (□) G = 6.0 $g \cdot l^{-1}$, P = 0 $g \cdot l^{-1}$; (△) G = 8.8 $g \cdot l^{-1}$, P = 0.2 $g \cdot l^{-1}$; (▽) G = 11.3 $g \cdot l^{-1}$, P = 0.2 $g \cdot l^{-1}$

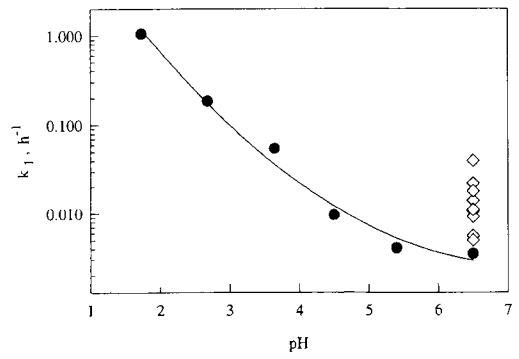

Fig. 6 Kinetic constants for the clavulanic acid in vitro decomposition reaction (fermentation supernatants) as a function of pH (●) and for the apparent in vivo decomposition reaction, observed during the stationary phase in different cultivations (◇)

compositions for both media are essentially the same or similar, at least. However, one has to take into account that the media with the extract were exposed to higher temperatures for considerably longer, namely about 1 h at 121°C for extract preparation and an additional 20 min for sterilisation of the filled bioreactor before inoculation. Thus, valuable nutrients and medium constituents could be decomposed. Furthermore, it is possible that in the presence of soy meal particles in the cultivation medium, the formation of hydrolases and particularly extracellular proteases by *S. clavuligerus* is induced, which continue degrading soy meal during the growth phase, and thus provide a steady supply of essential nutrients to the microorganisms.

A second major difference among the data given in Figs. 1–4 is certainly the appearance of CA and its profiles. Growth and CA generation occur obviously simultaneously in the trophophase and the CA concentration is seemingly constant in the stationary phase when the soy meal extract is applied (Figs. 1, 2). In contrast, when soy meal particles are present in the media (Figs. 3, 4), production of CA is fully supressed during fast growth. Instead, a pronounced idiophase is observed with a steady, approximately constant CA formation rate. These observations could confirm the predominance of regulatory inhibition or repression mechanisms exerted by high activity of the cell machinery, which, however, cease to be operative when the growth rate decreases.

High initial concentrations of glycerol seem to have no significant influence on the production of CA in the case of cultivations with the media containing soy meal extract. However, when the medium was enriched with soy meal, the attained CDW and product concentration were comparatively lower for a higher initial glycerol concentration. A simultaneous negative effect of the higher phosphate concentrations for the run shown in Fig. 4 during the experiment can be excluded, as the same cultivation with a lower initial concentration of phosphate yielded a comparable final product concentration (data not shown).

The rates of glycerol consumption were very similar for all experiments (σ, Table 1), independently of the different metabolic activities observed for the different medium compositions. The higher growth rates are then sustained only by the nutrients available from the soy meal and are not reflected by a higher consumption of glycerol during the growth phase. Surprisingly, the

consumption rates of glycerol in the stationary phase were 3.5 to 4 times higher than in the growth phase (σ, Table 1), although no noticeable growth or production activity takes place, especially in the cultivations with soy meal extract medium. Once more, this is an indication that the essential nutrients for growth are preferentially supplied by soy meal. Due to the low amounts of CA produced, any significant consumption of glycerol due to CA synthesis is hardly detectable.

During the stationary phase of the cultivations with soy meal extract medium, the CA production is apparently zero. As nutrients are still available, this finding is difficult to explain from the viewpoint of regulation. In all the cultivations with this medium the CA concentration profiles during the stationary phase were usually rather flat and showed positive and negative deviations which were larger than the experimental scatter of the CA determination. Furthermore, in the majority of runs carried out with the extract medium the CA concentration tended to decrease. Examples for this observation are shown in Fig. 5. On the other hand, in the cultivations with soy meal in the medium, the CA concentration steadily increased or remained constant during the late stationary phase.

The glycerol consumption rates in the stationary phase are too high to account only for maintenance energy. Therefore, one has to assume that glycerol is also used for the production of secondary metabolites such as CA, which was the only compound being determined. This raised the hypothesis that also in the cultivations with soy meal extract medium, CA production still progresses during the stationary phase, but is accompanied by simultaneous decomposition of CA. Product decomposition or re-consumption could also explain the decrease of CA concentrations, as shown in Fig. 5.

As indicated in Fig. 6, the kinetic constants for the in vivo inactivation of CA are considerably higher (two- to ten fold) than the in vitro decomposition (in the absence of cells). While acid-catalysed hydrolysis appears to be mainly responsible for the measured in vitro instability of CA, as indicated by the pH dependency of k_1 in Fig. 6, one or several additional mechanisms seem to be active in the CA degradation in the stationary phase of cultivations carried out with soy meal extract medium. It is understood that the release of an intracellular component (due to cell lysis) and the induced production and release of β-lactamase cannot explain the observed differences, as such exocellular compounds would also be present in the cell-free supernatants. The production of growth-protecting substances by growing cells when the formation of the product is simultaneously taking place is known to be an important mechanism by which antibiotic-producing species avoid suicide (Martín and Demain 1980). When the production phase occurs after the growth phase, the synthesis of these substances during the stationary phase does not occur, since protection of the growth is no longer necessary. In the case of β-lactams, these growth-protecting substances could be enzymes that modify the antibiotic structure, one example of which are β-lactamases. However, formation of β-lactamases by *S. clavuligerus* has not yet been observed (Doran et al. 1990). On the basis of the available experimental data one can therefore only speculate about the higher in vivo decomposition rates of CA. One possibility could certainly be re-consumption of CA by the organism to form other secondary metabolites, which were not detected, or the modification of its structure to detoxify the medium during the growth phase by cell-wall-retained enzymes.

A consistent and unambiguous explanation of the different CA profiles observed when using soy meal as such, or soy meal extract in the cultivation medium also cannnot be given. One possibility is that CA production may be higher than its decomposition in the soy meal medium, due to better nutrient supply than in case of the extract medium. A second explanation could be that the pattern of separated growth and production phase obtained with soy meal medium leads to no induction of the synthesis of CA-degradating (detoxifying) enzymes, whereas this induction occurs during the simultaneous growth and production patterns obtained with soy meal extract medium and causes the degradation of CA during the stationary phase. A third interpretation could be that the presence of soy meal particles in the culture may enable adsorption of CA which is thus protected from microbial attack. A similar effect was reported by Ivanitskaya et al. (1985), who observed improved CA production in the presence of activated charcoal particles.

Not taking into account the phenomenon of the in vivo CA degradation, it is clear that the cultivations with soy meal extract medium are advantageous from the viewpoint of productivity. CA productivity during the growth phase of the cultivation with medium containing soy meal extract and a low initial concentration of glycerol (Fig. 1) is 3 times higher than the productivity during the stationary phase of the cultivation with medium containing soy meal particles and low initial concentration of glycerol (Fig. 3). However, if the in vivo degradation of CA occurs and if the cause of this phenomenon is known, the production levels of CA could be enhanced by selecting medium compositions and operation conditions that minimise this effect.

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