The inhibitor formed by *Isochrysis galbana* and its effect on the growth of microalgae

(황색편모조류(*Isochrysis galbana*)에 의한 생육저해물질의 생성 및 미세조류 생육에 미치는 영향)

Yingying SUN[1], Changhai WANG*[1,2], Liqin SUN[2], Jing CHEN[2]

[1] Department of Bioscience and Biotechnology, Dalian University of Technology, Dalian 116024, P.R. China

[2] School of Ocean, Yantai University, Yantai 264005, P.R. China

Prof., Changhai WANG

School of Ocean, Yantai University

China

chwang2001@sina.com syy-999@163.com

Tel.: +86-535-6706288 Fax: +86-535-6706299

Abstract

The inhibitor, a kind of ethyl acetate crude extract, was isolated from the old culture liquid of *I.galbana.* Through measured the culture density, the contents of chlorophyll a, polysaccharide and protein in the cells, the inhibitory effect of the inhibitor on the growth of cells was investigated. The results showed that the inhibitor obviously inhibited the cell growth of three microalgae.

Keywords: Inhibitor, Ethyl acetate extract, *I. galbana, C. gracilis, C.vugaris*

1 Introduction

The growth of the unicellar microalgae declines in the late exponential growth phase and the cells gradually die. Ingenerally, the nutrition limitation or some stress conditions are considered as the key factors stopping the cell division however the effect of some inhibitory chemical compounds produced by microalgal cells themselves received little attentions. Imada *et al.* [1] found that the extracts from the old cultured medium of *S. costatum* had inhibitory effect on the growth of *S. costatum.* Sun *et al.* [2] also found that the auto-signals exited in the old culture of *H. pluvialis*would reduce the cell growth rate. Subsequently Imada *et al.* [3] isolated an inhibitor from the old cultured medium of *S. costatum,* and considered that it was probably an oxygenated metabolite of EPA (eicosapentaenoic acid). McGrattan *et al.* [4] recognized that the chlorellin reported and named by Pratt [5], that produced by *C. vulgaris* own and restrained its own growth, and considered as well as that was a kind of photooxygenated product of unsaturated fatty acid, whereas some researchers considered [6,7] that polyunsaturated acids themselves and not their degradation compounds were responsible for the growth inhibition of microalgal cells.

In this study, an ethyl acetate crude extract was isolated from the old culture liquid of *I. galbana*. To determine itsinhibitory activity, the effect of the inhibitor on the growth, intracellular contents of chlorophyll a, polysaccharide and protein of three microalgae of *I. galbana, C. gracilis* and *C. vugaris* were investigated.

2 Materials and methods

2.1 Microorganism

I. galbana, C. gracilis and *C. vugaris* were obtained from the Ocean University of China.

2.2 Cultures

All algal cells were cultured in 300 mL flasks containing 200 mL of autoclaved filtered seawater enriched with nutrients M-f/2 (Guillard and Ryther, 1962)[8]. Cultures were incubated for 14 days in shaker at 100 rpm and 23℃ with 1.10 $mWcm^{-2}$ illumination in a 16h:8h light-dark cycle.

Isochrysis galbana was cultured in different medium, which made of M-f/2 added different concentrations inhibitor from medium of *Isochrysis galbana*, respectivelly. The inhibitor concentrations added to M-f/2 is 50,100,150,200,250,300、350,400,450,500,550 and 600$\mu\ell$, respectivelly. And compared with that of inhibitor free medium f/2 as the control.

C. gracilis and *C. vugaris* were also cultured in medium as mentioned, which made of M-f/2 added 200 and 600$\mu\ell$ inhibitor from medium of *Isochrysis galbana*.

2.3 Isolation of inhibitor from *I. galbana* culture supernatant

1600 mL of *I. galbana* culture broth of 20 days old was centrifuged at 5000 rpm for 15 min; the supernatant was filtered through a fiber glass filter (GF/C,

the great eur-asia(Beijing) sci &development co., ltd, 9901026) of 0.45 μm. The filtrate pH was adjusted to 2-3 using 1 mol HCl and extracted three times with ethyl acetate. The extract was evaporated to 16 mL under reduced pressure at 40℃, and 13.6 g of yellow-brown oily liquid was obtained by this process, which was immediately stored at -4℃ refrigerator.

2.4 Growth measurement

The cell number is counted directly by a haemacytometer under microscope. The samples were taken after adding the inhibitorinto medium for 48 h and 96 h, respectively. Each sample is counted three times and three replicates for each sample.

Percentage of Inhibition (%) = (1-Cell density with inhibitors / cell density without inhibitors (control))100%.

2.5 Measurement of intracellular protein, polysaccharide and chlorophyll a

40 mL of culture broth was centrifuged at 3000 rpm for 10 min; pellet cells were immediately stored at -20℃ for further analysis.

Polysaccharide, protein and chlorophyll a were measured by the methods of anthrone, coomassie brilliant blue G250 and acetone respectively, as previously described by Wang [9]

Relative content (%) = Content with inhibitors/Content without inhibitors (control) ≠100%.

3 Results

3.1 Effect of inhibitor extract on the cell density, contents of polysaccharide, protein and chlorophyll a in *I. galbana*

When 50 $\mu\ell$ and 100 $\mu\ell$ of the inhibitor extract were added into the cultures for 48 h, the growth of *I. galbana* increased. However, in the range of 150 to 600 $\mu\ell$, the inhibitor extract exhibited strong inhibitory effect on the cell growth. When compared with the control after addition for 48 h, cell densities of treatment groups decreased to 0.876, 0.921, 0.944, 0.943, 0.584, 0.528, 0.629, 0.584 and 0.606 respectively (Fig.1). After 96 h, the inhibitory effect was more pronounced, cell densities oftreatment groups decreased to 0.464, 0.463, 0.391, 0.288, 0.268, 0.391, 0.494, 0.453 and 0.371 respectively (Fig.1). According to the data, the LC50 (half killed concentration) of cell density for the inhibitor at 96 h was 149 $\mu\ell$.

As shown in Fig.2, the pattern of chlorophyll a of *I. galbana* was similar to that of cell density. When the amount of inhibitor added from 200 to 600 $\mu\ell$, the relative contents of chlorophyll awere lower than the control, varied from 0.461 to 0.279. At 96 h LC_{50} value of the extract inhibitor for chlorophyll a was 210 $\mu\ell$. However, the phenomenon that a stimulative effect on the chemosynthesisof chlorophyll a was observed, when 50 and 150 $\mu\ell$ of inhibitor were added into the cultures.

The inhibitor showed noticeable inhibitory effect on the protein synthesis of cells, when 100 to 600 $\mu\ell$ of the inhibitor was added, the relative protein contents varied from 0.841 to 0.837 (Fig.2). Maximum relative protein content was found with the addition of 50 $\mu\ell$ extract, the extract had stimulative effect on the protein synthesis of *I. galbana* under low doses.

At the 96 h, the relative polysaccharide contents in all experiment groups were lower than that of the control, the range of the polysaccharide content was from 0.996 to 0.396 (Fig.2). Comparing with protein and chlorophyll a, polysaccharide was more sensitive to extract inhibitor, and its LC_{50} value for was 173 $\mu\ell$.

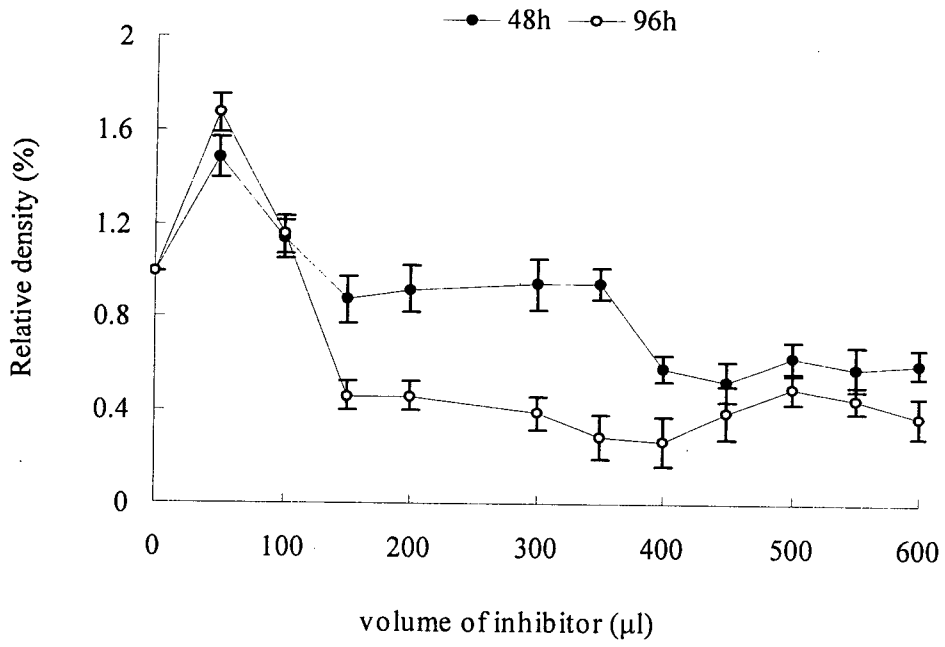

Fig.1. Effect of inhibitor on the cell density of *I. galbana*

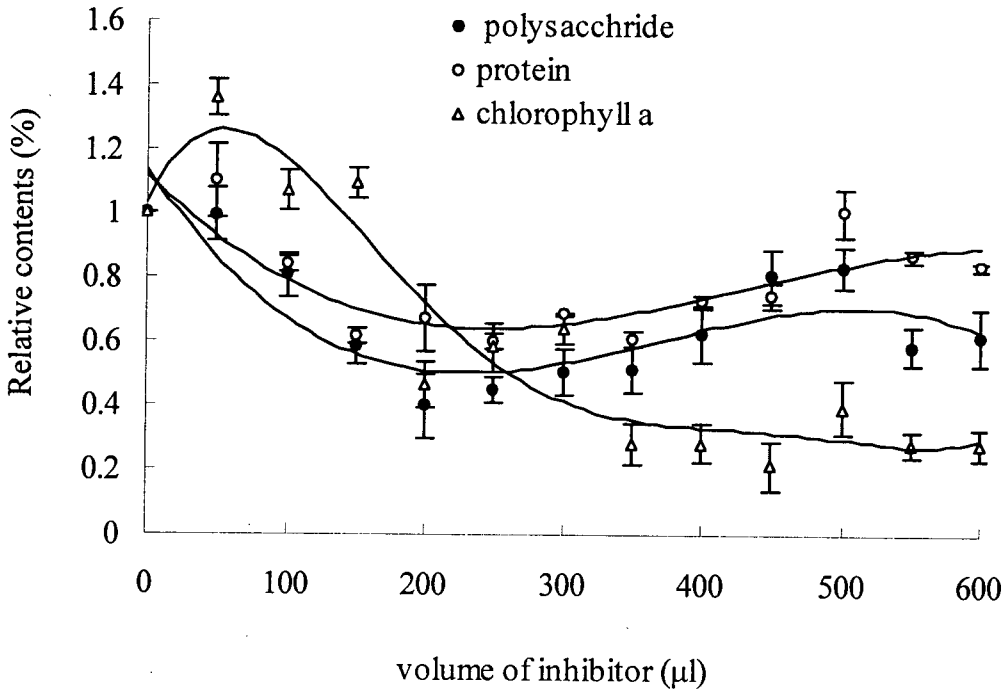

Fig. 2. Effect of inhibitor on the polysacchride, protein and chlorophyll a of *Isochrysis galban*

3.2 Effect of the extract inhibitor on the cell density, content of chlorophyll a, protein and polysaccharide in *C. gracilis* and *C. vugaris*

The cells of *C. gracilis* weresensitive to the extract inhibitor, and the relative cell densities of *C. gracilis* were 0.691 and 0.782 at the 48 h and 96 h respectively (Fig.3). Below the doses of 200 $\mu\ell$, the inhibitor had a distinct stimulative effect on synthesis of chlorophyll a whereas the synthesis of protein and polysaccharide were inhibited when the inhibitor was more than 200 $\mu\ell$, the relative protein and polysaccharide contents were 0.553 and 0.713, and nearly all biochemical process were inhibited at the dose of 600 $\mu\ell$ (Fig.4).

The inhibitor showed strong inhibitory effect on *C. vugaris* than that on *I. galbana* and *C. gracilis*. After added the inhibitor into cultures for 48 h, the relative cell densities of *C. vugaris* decreased to 0.405 and 0.274 at the dose of 200 $\mu\ell$, and the 48 h cell density LC_{50} for the inhibitor was less than 200 $\mu\ell$ (Fig.5). At the dose of 200 $\mu\ell$, the inhibitor had slight inhibitory effect on the synthesis of chlorophyll a, but it showed very strong inhibitory effect on the synthesis of protein and polysaccharide in *C. vugaris*. The relative content of protein and polysaccharide decreased to 0.339 and 0.109, when the inhibitor added to 600 $\mu\ell$, the relative content of chlorophyll a, protein and polysaccharide decreased to 0.674, 0.320 and 0.0968 respectively (Fig.6).

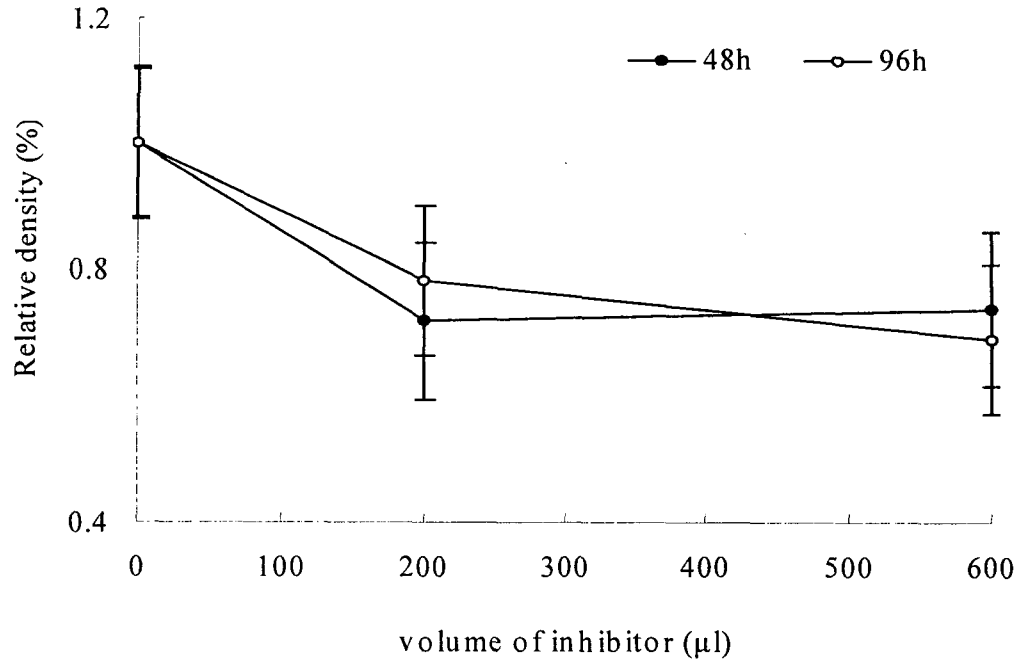

Fig. 3. Effect of the inhibitor on the cell density of *C.gracilis*

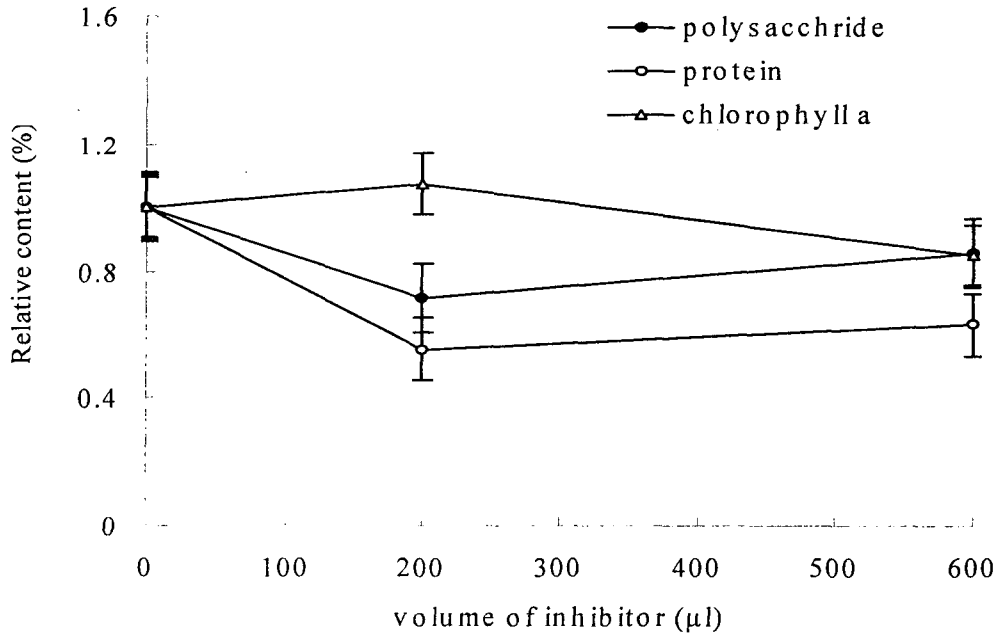

Fig. 4. Effect of the inhibitor on the polysacchride and protein and chlorophyll a of *C. gracili*

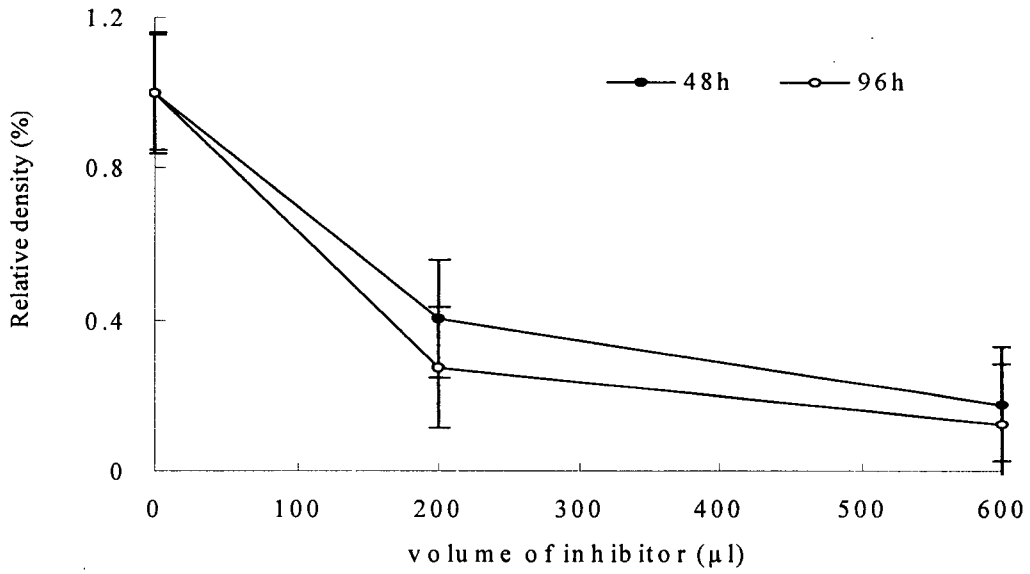

Fig. 5. Effect of the inhibitor on the cell density of *C. vugaris*

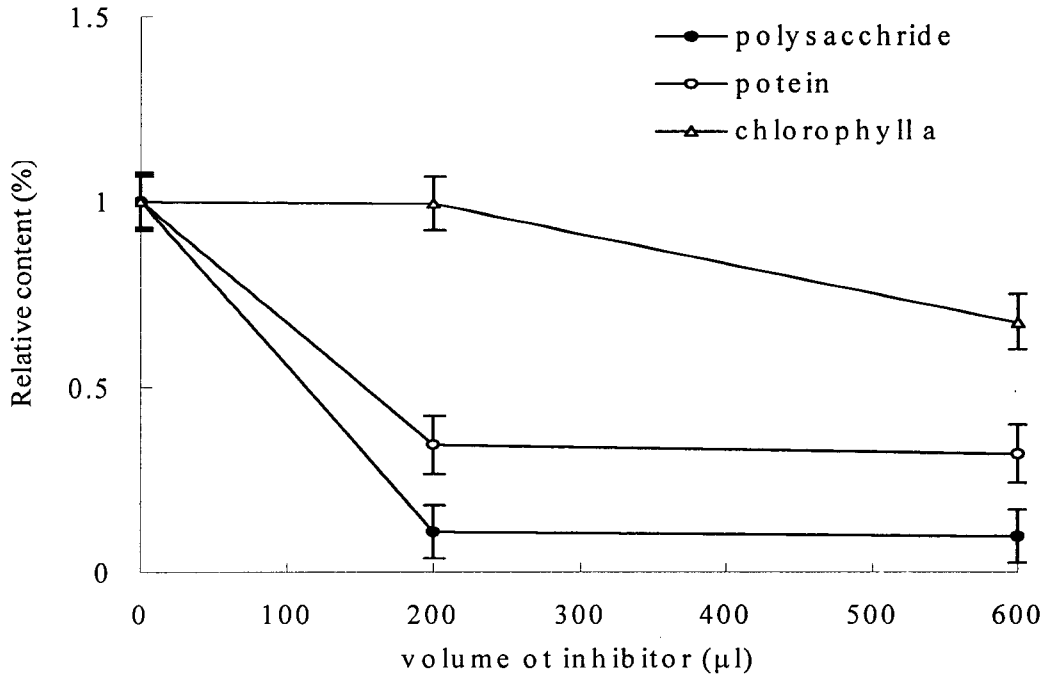

Fig. 6. Effect of the inhibitor on the polysacchride, protein and chlorophyll a of *C. vugaris*

4 Discussion

The results of this experiments suggest that *I. galbana* produces an inhibitor and release gradually into the medium in course of growth, restraining the cell growth and the synthesis of polysaccharide, protein and chlorophyll a. It accords with Fogg's report (Fogg and Hellebus, 1974) [10] mentioned above, namely, microalgae release manifold metabolize products into ambience in course of growth, thereinto include inhibitor.

Imada *et al* .[1]reported that autoinhibitor by *Skeletonema costatum* could restrain 6 species of phytoplankton, and pointed out that the autoinhibitors were accumulated in the cells from the exponential growth phase mostly up to the stationary phase, when the concentration beyond a lethal level, they were released into the medium to presumably attack the surviving cells.

Sun et al. [2] also reported that some unknown substances existed in the old culture of *H. pluvialis* would reduce the cell growth rate.

It is clearly that the lower levels of the extract inhibitor had stimulative effect on the cell density, polysaccharide, protein and chlorophyll a in all three microalgae tested. This results were in a way similar to that described by Beaumont and Newman [11]. And this phenomena was explained by the parlance described by Stebbing (1982) [12]. Stebbing had indicated that low levels of poisonreduced growth of marine microalgae. This phenomena named excited domino offect of poison by Stebbing was an excitive reaction in void poison instance. However all these parameters decreased at the higher extract inhibitor doses. The inhibitory effect was dosage-dependent on the inhibitor.

Table 1 showed that the extract inhibitor had different effect on the cell density, chlorophyll a, protein and polysaccharide of three algal strains. The sensitivity to the extract inhibitor declined in the order of *C. vugaris, I.galban* and *C. gracilis*. According to Imada *et al*[4], his experiments show that autoinhibitor by *Skeletonema costatum* could restrain 4 species of diatoms and 2 species of *chattonella* except for 2 species of dinoflagellates. It was obvious that the

sensitivities to inhibitor were different for different species of marine microalgae.

Table 1. Effect of the inhibitor on the cell density, chlorophyll a, protein and polysaccharide in *I. galban*, *C. gracilis* and *C. vugaris*

	added 200 $\mu\ell$ of the inhibitor			added 600 $\mu\ell$ of the inhibitor		
	I. galban	*C. gracilis*	*C. vugaris*	*I. galban*	*C. gracilis*	*C. vugaris*
cell density	0.464	0.782	0.274	0.371	0.691	0.126
chlorophyll a	0.461	1.074	0.997	0.279	0.853	0.674
protein	0.671	0.553	0.339	0.837	0.632	0.321
polysaccharide	0.397	0.713	0.109	0.616	1.186	0.097

The sensitivity of biochemical parameters in response to the inhibitor was also different for three microalgae. Polysaccharide synthesis was more significantly repressed in *C. vugaris* and *I. galban* than proteins and chlorophyll a, but protein synthesis was significantly inhibited in *C. gracilis*.

Although we could not obtain chemical compounds of inhibitor from the old culture liquid of *I. galbana*, but this experiments had proved that inhibitor exited in the old culture liquid of *I. galbana* and it could not only restrain the growth of three microalgae, but also restrained the biosynthesis of polysaccharide, protein and chlorophyll a in those microalagal cells.

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