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RESEARCH ARTICLE

Enhancement of Microbial Oil Production by Alpha-linolenic Acid Producing *Yarrowia lipolytica* Strains QU22 and QU137

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Abstract Microbial oils enriched in essential polyunsaturated fatty acids can be used as nutritional complements. In order to improve the lipid yields and lipid profiles of 2 *Yarrowia lipolytica* strains, the effects of medium components and culture conditions were investigated using shake flasks. Under optimized conditions (cultivation for 3 days at 25ºC and 150 rpm, using peptone as a nitrogen source and glucose as a carbon source), the biomass, lipid yield, and lipid content reached 6.75 g/L, 3.48 g/L, and 51.55% for *Y. lipolytica* QU22, and 6.85 g/L, 3.52 g/L, and 51.38% for *Y. lipolytica* QU137, respectively. Using a shaking speed of 150 rpm, unsaturated fatty acids constituted 87.96% (QU22) and 87.08% (QU137) of the total lipids produced by these strains, with 9.52 and 7.86% of alpha-linolenic acid, respectively. The strains are suitable candidates for fermentation processes involving essential polyunsaturated fatty acid (PUFA) production.

Keywords: yeast, oil, *Yarrowia lipolytica*, fatty acid profile

Introduction

Microorganisms that accumulate more than 20% lipids of their body weight are defined as oleaginous. In the last decade, there has been a growing interest in lipid producing yeasts due to accumulation of large amounts of lipids inside cells (1). Research progress in this field has shown that synthesis of lipids by these organisms can be partially controlled, and methods for assay and extraction of lipid molecules are now established (2). Only a small number of yeast species, approximately 30 out of more than 600 described species, are considered oleaginous. These yeasts are generally non-pathogenic and are able to synthesize and accumulate lipids of a composition similar to vegetable oils (3).

Natural manufacturing of healthy oils containing polyunsaturated fatty acids (PUFAs) is one of the major interests of lipid biotechnology. PUFAs are essential compounds that act as precursors of diverse metabolites, such as prostaglandins, leukotrienes, and hydroxy-fatty acids (4). Alpha-linolenic acid (ALA) is an important PUFAs due to beneficial effects on human health regarding cardiovascular disease, plasma lipid levels, and arrhythmia, and for anti-inflammatory and neuroprotective properties (5). ALA is considered essential for mammals, acting as a precursor of eicosapentaenoic (EPA) and docosahexaenoic acids (DHA). Limited dietary ALA sources have resulted in a search for alternative sources. Microorganisms able to produce this essential fatty acid may be able to supply ALA.

In a previous work (6), 86 yeast strains were initially screened using Nile red dye fluorescence in order to select potentially oleaginous taxa (7). Lipid profiles of 16 oleaginous strains belonging to the species *Yarrowia lipolytica* were analyzed (unpublished data) and strains QU22 and QU137 were selected due to production of alpha-linolenic acid (ALA). In this study, these 2 ALA producers were investigated in order to evaluate parameters that influence lipid production.

The oleaginous yeast *Y. lipolytica* is an extensively studied nonconventional yeast, that is species that are not closely related to *Saccharomyces cerevisiae*. Several technologies, including different fermentation configurations, have been

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applied for single-cell oil production by strains of *Y. lipolytica*, resulting in oils with an appealing fatty acid composition, such as enriched in essential polyunsaturated fatty acids, that could be used as nutritional complements (8,9). Groenewald *et al.* (10) reviewed the safety of *Yarrowia*-derived products and concluded that *Y. lipolytica* is a safe organism for use.

It has been extensively reported that cultivation conditions, such as temperature, pH, oxygen content, and concentration of trace elements, influence oil accumulation in microorganisms (1,11-15). Different nitrogen sources also influence oil production with both inorganic and organic nitrogen sources used for yeast cultivation (16,17). To achieve high levels of oil production, optimization of cultivation parameters is important. Thus, the aim of this study was to evaluate the influence of cultivation time, nitrogen source, incubation temperature, and shaking speed on the amount of total lipid and unsaturated fatty acid production by *Y. lipolytica* QU22 and QU137.

Materials and Methods

Microorganisms Yeast strains used were isolated from samples of artisanal cheese collected in Rio Grande do Sul, Brazil (6) in 2006, and kept freeze-dried (Liotop L 1001; São Carlos, Brazil) and frozen at –30°C (Indrel CLC300DAF Freeze System; Londrina, Brazil). The QU22 and QU137 strains were identified as *Y. lipolytica* based on sequencing of the D1/D2 region of 26S rDNA (data not shown). Specimens were deposited at the Collection of Microorganisms, DNA, and Cells of the Federal University of Minas Gerais (Minas Gerais, Rio Grande do Sul, Brazil), under the access numbers UFMG-CM-Y328 and UFMG-CM-Y341. The strains were maintained using monthly subculture on YEPD agar slants (2% glucose, 0.5% yeast extract, 1% peptone, 2% agar), and kept in a refrigerator at 10ºC.

Parameters tested for lipid accumulation Parameters analyzed were the cultivation time (24, 48, 72, 96, and 120 h), nitrogen source, including peptone (Himedia, Mumbai, India), tryptone (OrganoTechnie, La Courneuve, France), NH_4NO_3 (Dinâmica, São Paulo, Brazil), and $(NH_4)_2SO_4$ (Cromoline, Diadema, Brazil) at incubation temperatures of 20, 25, and 30ºC, and shaking speeds of 150, 200, and 250 rpm.

Culture conditions A pre-inoculum was prepared using a standard medium containing 10% glucose (Dinâmica), 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4 (Vetec, Rio de Janeiro, Brazil), and 0.05% $MgCl_2 \cdot 6H_2O$ (Nuclear, São Paulo, Brazil). Cells were grown to an optical density of 1.0 at 600 nm (Meter Spectrophotometer, São Paulo, Brazil) in a rotary shaker (IKA, KS 4000, Campinas, Brazil) at 150 rpm and 25ºC. A seed culture (1%) was inoculated into 500 mL flasks containing 150 mL of the experimental culture medium, which was similar to the standard medium described above, with substitution of the nitrogen source in corresponding assays. Cultivation was carried out in a rotary shaker (IKA, KS 4000) using a different temperature, shaking speed, cultivation time, and nitrogen source for each assay. Each 100 mL of medium had 0.1 g of nitrogen source, and each 1 g of peptone used in this study contained 0.12 g of nitrogen (based on supplier information). An amount of 1 g of tryptone, NH_4NO_3, and $(NH_4)_2SO_4$ contained 0.13, 0.35, and 0.21 grams of nitrogen, respectively.

Determination of biomass Cells were harvested by centrifugation at 5,000×*g* for 10 min (ZN06; Ciencor, São Paulo, Brazil), washed twice with distilled water, and dried on a drying oven at 105ºC (NI1500; Nova, São Paulo, Brazil) to a constant weight using an analytical balance (AY220; Shimadzu, São Paulo, Brazil). Biomass was expressed as g of dry biomass per L of medium (g/L).

Determination of total lipids Lipids were extracted from the biomass following the method of Bligh and Dyer (18). Dry biomass was suspended in chloroform/methanol (2:1, v/v) and cell lysis occurred in a Turrax homogenizer (ULTRA-TURRAX T18; IKA, São Paulo, Brazil) with cycles of 2 min and ice cooling for 1 min to avoid lipid heating. For determination of the total lipid content, solvents were evaporated at 105ºC (NI1500; Nova) to a constant weight. The lipid yield was expressed as g of lipid per L of medium (g/L) and the lipid content was expressed as a percentage of the lipid weight per dry biomass weight (% w/w).

Analysis of the lipid composition Lipids were transesteried following the method of Hartman and Lago (19), and fatty acid methyl esters (FAME) were analyzed using GC (series 6890N; Agilent Technologies, Santa Clara, CA, USA) equipped with a 100 m fused-silica capillary column and a 0.2 μm film thickness (Supelco; Sigma-Aldrich, St. Louis, MO, USA). The column was heated to 35ºC for 2 min with a gradual increase of 10ºC per min until 150ºC, followed by holding for 2 min, then a further increase of 2ºC per min up to 200ºC, followed by holding for 2 min, and again increased at 2ºC per min up to 220ºC, followed by holding for 21 min. The total run duration was 73.5 min. Nitrogen was used as carrier gas at 0.9 mL/min. The sample volume injected in split mode (1:100) was 1 μL. The temperature used for the flame ionization detector (FID) was 280ºC. Fatty acids were quantified and identified by comparison of peak areas and retention times with

reference standards (Supelco 37 FAME Mix; Sigma-Aldrich). Results were expressed as a percentage of the total area of the identified fatty acid.

Statistical analysis All measurements were made at least in duplicate. Results were analyzed using an analysis of variance (ANOVA) and *post hoc* comparisons were made using Tukey's test ($p<0.05$). Statistical analyses were carried out using Statistics (version 9.1; Statsoft., Inc., Tulsa, OK, USA).

Results and Discussion

Y. lipolytica strains QU22 and QU137 were previously shown to produce ALA (unpublished data), an essential PUFAs that has limited sources in the human diet. This study evaluated the influence of cultivation time, nitrogen source, incubation temperature, and shaking speed on production of total lipids and PUFAs by these strains with an aim for use of QU22 and QU137 as alternative sources of ALA.

Effect of cultivation time The biomass, lipid yield, and lipid content for both QU22 and QU137 gradually increased with time until the 3rd day ($p<0.05$). From the 3rd to the 5th days, no significant increase ($p<0.05$) of the analyzed parameters was observed (Fig. 1). On the 3rd day, the biomass of *Y. lipolytica* QU22 was 5.52 g/L and the lipid yield was 2.21 g/L, corresponding to a lipid content of 40.04% (w/w). Similarly, *Y. lipolytica* QU137 produced 5.26 g/L of biomass and 2.12 g/L of lipids, corresponding to a lipid content of 40.30% (w/w). These biomass values were comparable to other published results, but the *Y. lipolytica* strains used in this study achieved higher lipid yields (4-36%) than other reported strains using glucose as a carbon source (20-23). Engineering the biosynthetic pathways resulted in an increase in the *Y. lipolytica* lipid content to 61.7% with glucose as a carbon source (24). A high lipid yield and lipid content were also produced by *Y. lipolytica* using other carbon sources. Tsigie *et al.* (25) reported a lipid yield of 6.68 g/L, corresponding to a lipid content of 58.5%, when sugarcane bagasse was used as carbon source. Papanikolaou and Aggelis (26) reported high lipid yields (up to 3.5 g/L) and a lipid content of 43% from highly aerated continuous cultures using industrial glycerol.

A decrease in the lipid content after a long period of cultivation has been reported, suggesting that oleaginous microorganisms use accumulated lipids for cell proliferation following a decrease in available glucose during the stationary phase (13,27,28). The cultivation time in this study was set at 3 days for subsequent analysis based on previous reports and no significant increase in lipid accumulation observed after the 3rd day.

Effect of the nitrogen source on biomass, lipid content, and fatty acid composition A significantly ($p<0.05$) larger biomass was reached using tryptone as a nitrogen source for both strains, compared with other sources (Table 1) peptone, $(NH_4)_2SO_4$, and NH_4NO_3 were poor nitrogen sources resulting in low biomass and lipid content values, probably due to formation of inhibitor acids (HNO_3) (25). $(NH_4)_2SO_4$ is a commonly used nitrogen source for cellular lipid accumulation (26,29,30). However, in this study, organic nitrogen sources were more efficient for biomass production and lipid yield. Therefore, the best nitrogen source should be used for each micororganism.

Although tryptone was the best nitrogen source for cell growth, it was not the most efficient for lipid accumulation (37.47% for QU22 and 38.04% for QU137). Lipid contents using peptone were 51.55 and 51.38%, respectively. Wu *et al.* (13) and Tsigie *et al.* (25) reported that peptone was the best nitrogen source for lipid accumulation using *Trichosporon capitatum* and *Y. lipolytica* Po1g, respectively. Zhu *et al.* (28) reported a lipid content of 62.4% when peptone was

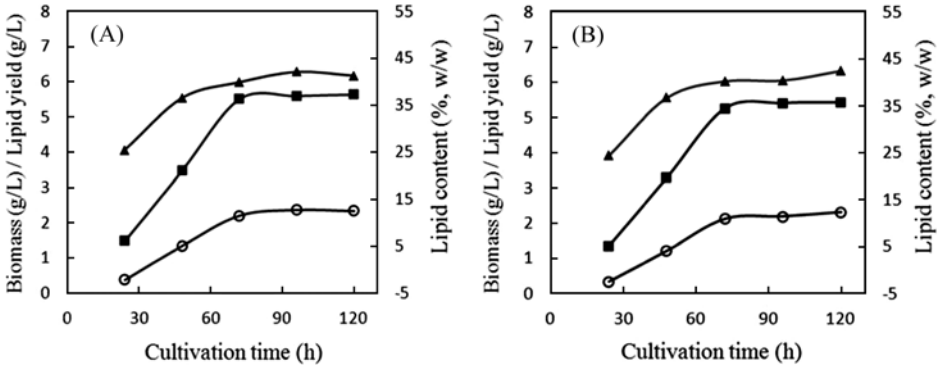

Fig. 1. Time course of cell growth and lipid accumulation of *Y. lipolytica* QU22 (A) and *Y. lipolytica* QU137 (B) at 25°C, 150 rpm. Medium composition: 10% glucose, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4 and 0.05% $MgCl_2 \cdot 6H_2O$. (■), biomass; (O), lipid yield; (▲), lipid content

Table 1. Effect of different nitrogen sources, incubation temperatures, and shaking speeds on cell growth and lipid accumulation by *Y. lipolytica* QU22 and *Y. lipolytica* QU137[1)]

Strain	Parameter	Biomass (g/L)	Lipid yield (g/L)	Lipid content (%,w/w)
	Nitrogen source[2)]			
QU22	Peptone	6.75±0.22^{b}	3.48±0.25^{a}	51.55
	Tryptone	8.7±0.44^{a}	3.26±0.15^{a}	37.47
	NH_4NO_3	1.14±0.06^{d}	0.03±0.02^{c}	2.63
	$(NH_4)_2SO_4$	5.38±0.13^{c}	2.12±0.04^{b}	39.41
QU137	Peptone	6.85±0.12^{b}	3.52±0.38^{a}	51.38
	Tryptone	8.99±0.14^{a}	3.42±0.2^{a}	38.04
	NH_4NO_3	0.89±0.12^{d}	0.02±0.01^{c}	2.25
	$(NH_4)_2SO_4$	5.05±0.05^{c}	1.97±0.12^{b}	39.01
	Temperature (°C)[3)]			
QU22	20	6.01±0.02^{a}	3.1±0.16^{a}	51.58
	25	6.33±0.31^{a}	3.06±0.05^{a}	48.34
	30	5.88±0.15^{a}	2.9±0.04^{a}	49.32
QU137	20	6.3±0.13^{ab}	3.14±0.12^{a}	49.84
	25	6.01±0.11^{b}	3.1±0.08^{a}	51.58
	30	6.58±0.04^{a}	2.67±0.17^{a}	40.58
	Shaking speed (rpm)[4)]			
QU22	150	5.62±0.28^{a}	2.46±0.45^{a}	43.77
	200	5.38±0.24^{a}	1.99±0.15^{a}	36.99
	250	5.9±0.03^{a}	2.51±0.11^{a}	42.54
QU137	150	5.3±0.12^{a}	2.46±0.01^{b}	46.42
	200	5.81±0.05^{a}	2.84±0.06^{a}	48.88
	250	5.27±0.23^{a}	2.15±0.08^{c}	40.80

[1)]All values are mean±standard deviation (SD) of triplicates. Means among the same strain within a column with no common superscript letter are significantly different ($p<0.05$). All cultures were performed for 72 h.
[2)]Conditions: 25°C, 150 rpm. Medium composition: 10% glucose, 0.1% KH_2PO_4, 0.05% $MgCl_2 \cdot 6H_2O$, and a 0.1% nitrogen source.
[3)]Conditions: 150 rpm and different temperatures. Medium composition: 10% glucose, 0.1% KH_2PO_4, 0.05% $MgCl_2 \cdot 6H_2O$, and 0.1% peptone.
[4)]Conditions: 25°C and different shaking speeds. Medium composition: 10% glucose, 0.1% KH_2PO_4, 0.05% $MgCl_2 \cdot 6H_2O$, and 0.1% peptone.

used as nitrogen source by *T. fermentans.*

Fatty acid compositions of strains QU22 and QU137 were investigated using peptone, tryptone, and $(NH_4)_2SO_4$ as nitrogen sources (Table 2). The resulting yeast oil contained mostly palmitic acid, oleic acid, linoleic acid, and ALA. *Y. lipolytica* is considered to be naturally deficient in ALA, but this fatty acid has been reported as 1% of the fatty acid profile (20). Other yeast species similarly do not produce high quantities of ALA. Galafassi *et al.* (31) found 2.02% of ALA when glucose was used as a carbon source by *Rhodotorula graminis*, while Èertík *et al.* (32) reported 2.8% for *R. glutinis*. Large quantities of ALA produced by *Y. lipolytica* have been reported after genetic engineering (33). In this study, QU22 and QU137 produced 13.06% (QU22) and 6.88% (QU137) of ALA using peptone without any genetic manipulation.

Changes in the quantity of each fatty acid were identified with variation of the analyzed parameters. When peptone and $(NH_4)_2SO_4$ were used as nitrogen sources, fatty acid profiles were similar with total unsaturated fatty acids of 89.99 and 88.17% (QU22) and 86.02 and 88.87% (QU137), respectively. When tryptone was used, the amount of total unsaturated fatty acids decreased to 65.92% (QU22) and 73.36% (QU137), and ALA was not identified. Total amounts of MUFAs and PUFAs vary among oleaginous yeasts. Lin *et al.* (12) reported total MUFAs of 55.9% and PUFAs of 2.7% using *Lipomyces starkeyi*. Huan *et al.* (34) reported total MUFAs and PUFAs of 39.4 and 12.5%, respectively, using *T. coremiiforme*. Even among different strains of *Y. lipolytica* with different culture conditions, total MUFAs and PUFAs can vary. Beopoulos *et al.* (20) reported total MUFAs of 34% and PUFAs of 52% using *Y. lipolytica.* Papanikolaou *et al.* (21) reported total MUFAs and PUFAs values of 78.3 and 5.3%, respectively, using *Y. lipolytica.*

Although tryptone is a good nitrogen source for biomass production, it was not the best for production of unsaturated fatty acid-enriched oil, which was the aim of this study. Peptone was the most appropriate nitrogen source for PUFAs production, with total PUFAs amounts of 45.48% (QU22) and 28.81% (QU137). For this reason, and considering that peptone was the best nitrogen source for total lipid accumulation (Table 1), peptone was used for subsequent analyses.

Table 2. Effect of different nitrogen sources on fatty acid profiles of *Y. lipolytica* QU22 and *Y. lipolytica* QU137[1)]

Strain	Nitrogen source	Palmitic acid	Oleic acid	Linoleic acid	Alpha-linolenic acid
QU22	Peptone	9.25±1.12[b2)]	45.21±1.01[ab]	31.72±0.58[b]	13.06±0.55[a]
	Tryptone	31.58±0.95[a]	48.69±2.05[a]	17.23±0.68[c]	ND**
	$(NH_4)_2SO_4$	8.11±0.55[b]	36.55±0.32[c]	41.58±1.65[a]	10.04±1.23[b]
QU137	Peptone	11.35±1.55[b]	57.21±0.65[bc]	21.93±1.26[a]	6.88±0.83[a]
	Tryptone	26.77±1.21[a]	66.78±1.24[a]	6.58±0.79[b]	ND**
	$(NH_4)_2SO_4$	10.97±1.32[b]	61.23±0.26[b]	21.55±0.79[a]	6.09±1.37[a]

[1)]Only the main fatty acids are shown. Results are percentages. All values are mean±SD of duplicates.
[2)]Means among the same strain within a column with no common superscript letter are significantly different ($p<0.05$). All cultures were done for 72 h at 25°C, 150 rpm. Medium composition: 10% glucose, 0.1% KH_2PO_4, 0.05% $MgCl_2 \cdot 6H_2O$, and a nitrogen source of 0.1%.
[3)]**Not detected

Table 3. Effect of shaking speed on fatty acid profiles of *Y. lipolytica* QU22 and *Y. lipolytica* QU137[1)]

Strain	Shaking speed (rpm)	Palmitic acid	Oleic acid	Linoleic acid	Alpha-linolenic acid
QU22	150	9.42±0.21[b2)]	43.65±1.24[b]	34.79±1.57[a]	9.52±2.02[a]
	200	8.34±1.38[b]	43.39±0.21[b]	34.87±1.67[a]	10.59±0.74[a]
	250	14.21±0.58[a]	48,89±1,82[a]	28.87±0.78[b]	4.88±1.23[b]
QU137	150	11.97±0.83[bc]	59.54±1.85[b]	19.68±1.25[a]	7.86±1.71[a]
	200	9.55±1.87[c]	61.32±0.59[b]	21.77±0.58[a]	6.89±0.81[a]
	250	13.87±0.52[a]	64.99±2.09[a]	16.78±1.86[b]	2.87±1.11[b]

[1)]Only the main fatty acids are shown. Results are percentages. All values are mean±SD of duplicates.
[2)]Means among the same strain within a column with no common superscript letter are significantly different ($p<0.05$). All cultures were done for 72 h at 25°C and different shaking speeds. Medium composition: 10% glucose, 0.1% KH_2PO_4, 0.05% $MgCl_2 \cdot 6H_2O$, and 0.1% peptone.

Effect of temperature and shaking speed on biomass, lipid content, and the fatty acid composition Incubation temperatures of 20, 25, and 30ºC were analyzed. A significantly ($p<0.05$) higher biomass was produced at 30ºC by QU137, compared with other temperatures, and no significant ($p<0.05$) differences were observed for biomass production values by QU22 at different temperatures (Table 1). No significant ($p<0.05$) differences were found for lipid yields for both strains among the 3 incubation temperatures used (Table 1). As either high or low temperatures are not desirable in industrial processes due to high costs and energy requirements, a broader range of temperature was not tested, and 25ºC was used in subsequent analyses.

There were no significant ($p<0.05$) differences in biomass production at different shaking speeds for both strains (Table 1). The lipid yield for strain QU22 produced similar results, but a higher lipid yield was obtained for strain QU137 when a shaking speed of 200 rpm was used. Fatty acid composition profiles among samples under different shaking speeds were analyzed (Table 3). At a shaking speed of 250 rpm, the amount of the saturated fatty acid palmitic acid increased significantly ($p<0.05$) to 14.21% for QU22) and to 13.87% QU137, compared with other speeds. There were also increases in the amounts of the monounsaturated fatty acid (MUFA) oleic acid with values of 48.89% for QU22 and 64.99% for QU137. On the other hand, the amount of total PUFAs decreased significantly ($p<0.05$) with shaking to an ALA content of 4.88% for QU22 and 2.87% for QU137. No significant ($p<0.05$) difference was found in the amounts of total PUFAs between 150 and 200 rpm. Therefore, 150 rpm was used as the shaking speed for subsequent analyses.

The oxygen level is an important factor that influences the fatty acid profile of oleaginous microorganisms, and both high and low oxygen saturations can be detrimental to the lipid yield and composition (35,36). Although the total biomass did not show variations between different shaking speeds in this study, the fatty acid profiles had a definite trend. A high shaking speed was not favorable for unsaturated fatty acid formation by *Y. lipolytica* QU22 and QU137 strains.

Depending on medium and culture conditions, fatty acid profiles of lipids accumulated by *Y. lipolytica* can change. Fontanille *et al.* (37) reported totals of saturated and unsaturated fatty acids of 61.6 and 38.4%, respectively, when a mixture of glucose and volatile fatty acids was used as carbon source. Katre *et al.* (24) reported significant differences among 5 *Y. lipolytica* strains grown on glucose where one strain produced 95.2% of unsaturated fatty acids and another produced only 29.4%. Under the optimized culture conditions of this study (cultivation for 3 days at 25ºC and 150 rpm, using peptone as a nitrogen source and glucose as a carbon source), amounts of total unsaturated fatty acids were 87.96% for QU22 and 87.08% for QU137, similar to results reported by Papanikolaou *et al.* (22)

(86.7%) using glucose as a carbon source and $(NH_4)_2SO_4$ as a nitrogen source for lipid accumulation by *Y. lipolytica*.

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