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BIODEGRADATION OF SOY BIODIESEL BY *PSEUDOMONAS AERUGINOSA* AND *PICHIA GUILLIERMONDII*

*Francisca Pessoa de França[1], Denise Mesquita Vieira[1] and Eduardo Homem de Siqueira Cavalcanti[2]
[1]Universidade Federal do Rio de Janeiro, Escola de Química - Centro de Tecnologia - Av. Athos da Silveira Ramos, nº 149, Bloco E, Sala 109 - Ilha do Fundão – Rio de Janeiro/RJ - 21941-909
[2]Instituto Nacional de Tecnologia, Av. Venezuela, 82, Sala 608, Saúde, Rio de Janeiro - 20081-312, Brasil

ABSTRACT

The biodegradation of soy biodiesel by two microorganisms isolated from fields contaminated with crude oil, *Pseudomonas aeruginosa* and *Pichia guilliermondii*, were compared using initial pH values of 4.0, 5.0 and 7.0 and initial biodiesel concentrations of 10, 20 and 30%. *Pseudomonas aeruginosa* was most efficient at pH 7.0, presenting degradation of 80, 33.9 and 25.7% of the respective biodiesel concentrations of 10, 20 and 30%. *Picchia guilliermondii* showed only a 31.2% reduction in medium containing 10% of the fuel at pH 7.0. Rapid degradation of soy biodiesel by *Pseudomonas aeruginosa* showed that the biodegradation of biodiesel depends on the degree of contamination and initial pH.

Keywords: Biodiesel, biodegradation, biofuel.

INTRODUCTION

Global efforts to reduce the emission of greenhouse gases into the atmosphere based on the Kyoto Protocol are a stimulus for technological research into the production of biofuels in Brazil. One of the most promising alternatives is biodiesel, consisting of a mixture of methyl esterified fatty acids derived from the transesterification of vegetable oils and animal fats, making biodiesel a clean and renewable fuel.

The search for alternative fuels is principally stimulated by high petroleum prices and world reserve projections, which have prompted the development of several studies with this objective (Leung *et al*., 2006; Pinto *et al.*, 2005). To be considered potentially important, an alternative fuel must present certain characteristics, including competitiveness, compatibility with existing motors, economic viability and environmental soundness (DeMello *et al.*, 2007). Biodiesel possesses these characteristics and its use is desirable since the raw material is abundantly available in the form of edible and non-edible oils, and the fuel itself is biodegradable and free of sulfur and aromatics (Kim *et al.*, 2004). Biodiesel is defined as an alternative fuel derived from renewable biological sources, and is produced by the transesterification of oils using methanol or ethanol in order to reduce its viscosity (Demirbas, 2008). In Brazil, biodiesel entered the national energy matrix in 2005, when, by law, 2% biodiesel was to be mixed with petrodiesel in 2008, reaching a 5% mixture by 2013. This will save Brazil an estimated US$152 million (Guarieiro *et al.*, 2008). However, the production of biodiesel generates glycerol in the proportion of six liters for each ton of biodiesel, and depending on the point of view, this large quantity of glycerol can be beneficial or cause a very significant problem (Engelhaupt, 2007). Although biodiesel has been publically known for over 100 years, very few studies on the degradation and biodegradation of biodiesel have been done. It is known that temperature elevation and exposure to air can increase the degradation of biodiesel when stocked (Leung *et al.*, 2006). During microbial degradation, fatty acid methyl esters are degraded at the same rate as n-alkanes, with some components degrading faster than others, and the entire mixture being consumed in weeks under laboratory conditions (DeMello *et al.*, 2007).

Given the importance of damages related to the biodeterioration and biodegradation of biodiesel, it is necessary to isolate microorganisms that develop during storage and understand their mechanisms in order to elaborate strategies that will allow the product to continue to be commercially viable. This study aimed to verify the biodeterioration of soy biodiesel by two microorganism species in synthetic effluents contaminated with different levels of the fuel.

MATERIALS AND METHODS

Microorganisms

The yeast *Picchia guilliermondii* and bacterium *Pseudomonas aeruginosa*, both isolated from an area impacted with crude oil, were used. After growth in Sabouraud Agar (Merck, Darmstadt, 105438) and Nutrient Agar (Merck, Darmstadt, 105450), respectively,

*Corresponding author email: fpfranca@eq.ufrj.br

the microorganisms were stored at 5±1°C. Prior to the experiments, the microorganisms were adapted to the biodiesel by successive cultures in mineral media in which biodiesel was the only carbon source.

Mineral Media

The media had the following composition, in g/l: 0.5 KH_2PO_4; 1.4 Na_2HPO_4; 1.0 NH_4NO_3; 0.1 $MgSO_4$; 0.02 $CaCl_2$ and 0.03 $MnSO_4$. The media pH was calibrated to pH 7.2±0.4.

Experiments

An experimental design was performed with the objective of studying the possible effects of the initial pH values and the initial concentrations of the biodiesel in the medium on the biodegradation activity of the microorganisms.

Experimental Design

Studies applying an experimental design have been done with the objective of optimizing the process parameters (Tanyildizi *et al.*, 2005; Abdel-Fattah, 2002; Abdel-Fattah and Olama, 2002). The best way to work with more than one parameter is through factorial experiments. This strategy consists of varying all of the factors simultaneously, allowing for definition of the effects of each factor in the system, as well as some possible interactions between them (Barros Neto *et al.*, 1995). For this study, a complete 3^n factorial design with a central point was used, where *n* is the number of factors and 3 is the number of levels developed. Nineteen tests were conducted, comprising eight conditions in duplicate, with a central point performed in triplicate (Calado and Montgomery, 2003; Montgomery, 1997; Barros Neto *et al.*, 1995; Box *et al.*, 1978)**.**

Biodiesel Biodegradation Studies

The experiments were done in 500mL Erlenmeyer flasks, in which 100mL of media was inoculated with the respective microorganisms. The flasks were agitated in an orbital shaker at 37±1°C and 300 rpm (Tecnal, TE-420, Brazil).

Experiments were performed according to the experimental design using a combination of three initial pH values, 4.0, 5.0 and 7.0, and three initial biodiesel concentrations, 10, 20 and 30% v/v.

Studies were done in duplicate and the average of the results is reported. Negative controls without the microorganisms were also performed. Each experiment had a nine-day duration, after which the final pH was determined, along with the biomass and final biodiesel concentration.

Quantifications

Values of pH were measured using a pH meter (Digimed, DMPH-1, Brazil). Biodiesel was quantified using gas chromatography (Shimadzu, GC-2014, Japan). The measurements were done in the range of lauric acid and linoleic acid esters (Standards UNE– EN14103).

RESULTS AND DISCUSSION

Table 1 shows the average results of biodiesel biodegradation by *Pseudomonas aeruginosa*. After analysis of the final biodiesel concentration in relation to the initial fuel quantity and the initial pH of the medium, we observed that the consumption of esters by *Pseudomonas aeruginosa* was more evident when the level of fuel in the medium was 10% and the pH was 7.0, as shown in table 1. At pH values of 4.0 and 5.0, the biodegradation was significantly reduced. With an increase in biofuel concentration, there was a reduction in degradative activity that was proportional to the reduction in pH of the medium.

When analyzing table 1, the program Design-Expert (Northwest Analytical, Inc., NWA) suggested that the quadratic model provided the best fit for this complete 3^2 design, as given by the equation:

$$Y=\beta_0+\beta_1 A+\beta_2 B+\beta_{12} AB+\beta_{11} A^2+\beta_{22} B^2+\varepsilon \quad (1)$$

where Y is the response, β_0 is the overall mean effect, A (pH) and B (initial biodiesel) are the controlled input variables, β_1 and β_2 are the effects of the biofuel concentration, β_{12} is the interaction effect, β_{11} and β_{22} are the quadratic effects and ε is the error component.

Table 2 shows the analysis of variance for biodiesel biodegradation by *Pseudomonas aeruginosa*. Using this microorganism, an encoded model was obtained, as described in equation (2):

$$\text{Biodegradation by } \textit{Pseudomonas aeruginosa} \ (\%) = +22.58 + 6.32A - 33.43B + 1.39A^2 + 26.92B^2 + 3.33AB \quad (2)$$

The values of F were compared to tabulated values at the 95% confidence level. If the value of F was greater than the tabulated one, the non-influence hypothesis (null) was rejected, meaning that the factor significantly affected the rate of emulsification. Values of p less that 0.05 indicated significance, showing that the model was valid and that the terms A and A^2 influenced the response.

The adopted model was evaluated through a normal probability plot of the residues shown in figure 1, with the values near the line indicating the validity of the proposed model. The squared correlation coefficient (r^2) indicated that 99.5% of the data variability fit to the model. The adopted model was also evaluated through the normal probability plot of the waste, as shown in Figure 1, with the values near the line indicating the validity of the proposed model.

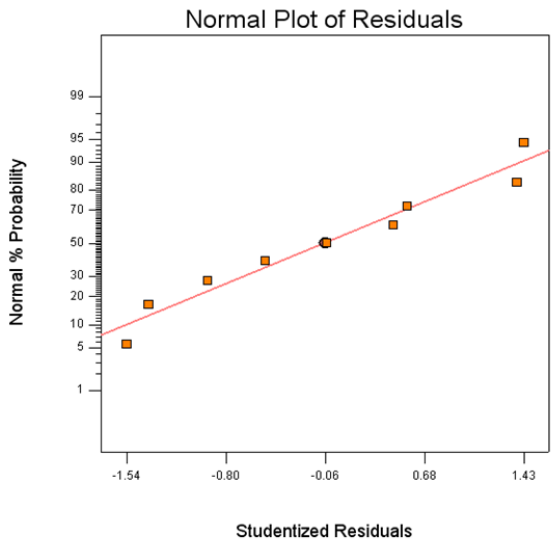

Fig. 1. Normal probability plot of the residuals from the model of biodiesel degradation by *Pseudomonas aeruginosa*.

The areas where biodegradation reached maximum values can be visualized in the contour plot in figure 2. It should be noted that from the boundary curves, the biodegradation increased when variable B (initial biodiesel) decreased from a higher level (30%) to a lower level. There was no significant influence of pH. This was verified by the perturbation plot (Fig. 3), which showed that there was a significant biodegradation decrease when B (initial biodiesel) moved from a higher level to a lower level.

In relation to the pH values, the behavior of *Picchia guilliermondii* (Table 1) was the inverse of that obtained for *Pseudomonas aeruginosa*; the highest biodegradation was approximately 27% at pH 4.0 with 10% biodiesel. Therefore, the degree of biodegradation was much less than the 80% value found for *Pseudomonas aeruginosa*.

In all experiments, the pH values decreased a trend that was more visible in the experiments conducted with yeast. While analyzing the results obtained with *Picchia guilliermondii* (Table 1), the Design-Expert program suggested a linear model as the best adjustment for the

Table 1. Biodiesel degradation by *Pseudomonas aeruginosa* and *Picchia guilliermondii* as a function of the initial pH.

pH	Initial biodiesel concentration (%)	Biodegradation (%)	
		Pseudomonas aeruginosa	*Picchia guilliermondii*
4	10	82.2	31.2
5	10	83.5	22.9
7	10	85.0	12.5
4	20	14.1	14.1
5	20	20.5	17.1
7	20	34.0	14.6
4	30	10.5	26.8
5	30	11.7	16.2
7	30	25.7	12.2

Table 2. Analysis of variance for biodiesel degradation by *Pseudomonas aeruginosa*

Factor	SS[a]	DF[b]	MS[c]	*F*-value	*p*
Model	8611.93	5	1722.39	112.92	0.0013
A	240.03	1	240.03	15.74	0.0286
B	6587.89	1	6587.89	431.88	0.0002
AB	2.93	1	2.93	0.19	0.6907
A^2	1449.01	1	1449.01	94.99	0.0023
B^2	45.91	1	45.91	3.01	0.1812
Error	45.76	3	15.25		
Total SS	8657.69	8			

A: pH; *B*: initial concentration of biodiesel
[a] Sum of squares, [b] Degrees of freedom, [c] Medium square

complete 3^2 design, as given by equation (3):

$$Y=\beta_0+\beta_1 A+\beta_2 B+\varepsilon \quad (3)$$

Where, Y is the response, β_0 is the overall mean effect, A (pH) and B (initial biodiesel) are the controlled input variables and β_1 and β_2 are the effects of the levels.

Table 3 shows the analysis of the variability of biodiesel biodegradation by *Picchia guilliermondii*. From these results, an encoded model was obtained, as described in equation (4):

$$\text{Biodegradation by } \textit{Picchia guilliermondii} \ (\%) = +18.02 - 5.30A - 1.91B \quad (4)$$

The analysis of variance (Table 3) showed that the model did not present significance at the 95% confidence level. This means that the phenomenon cannot be described by this model; however, it does not invalidate the experiment. This was also verified by the perturbation plot (Fig. 4), where the two independent variables similarly influenced the biodegradation of biodiesel by *Picchia guilliermondii,* because there was a reduction in biodegradation when the variables moved from a lower to a higher level.

The squared correlation coefficient (r^2) indicated that only 55.3% of the data variability fit the model.

Pseudomonas aeruginosa and *Picchia guilliermondii* presented better results of biodiesel degradation at pH 7.0 and 4.0, respectively. Figures 5 and 6 are example chromatograms obtained after actuation of these microorganisms in media containing 10% biodiesel, where maximum consumptions of 8.39% and 6.88% of the fuel were observed. In both situations, we observed the presence of palmitic, stearic, oleic and linoleic acids.

In all experiments a pH decrease was observed, being most visible in the experiments performed with yeast. De Mello *et al*. (2007) affirmed that fatty acid methyl esters found in biodiesel are rapidly degraded by microbial strains at the same rate as n-alkanes. According to the authors, this is due to the molecular structure, which contains a higher number of saturated carbons than unsaturated carbons.

Table 3. Analysis of variance for biodiesel degradation by *Picchia guilliermondii.*

Factor	SS[a]	DF[b]	MS[c]	*F*-value	*p*
Model	196.37	2	98.19	3.43	0.1016
A	174.59	1	174.59	6.10	0.0485
B	21.79	1	21.79	0.76	0.4166
Error	171.83	6	28.64		
Total SS	368.21	8			

A: pH; *B*: initial concentration of biodiesel
[a] Sum of squares, [b] Degrees of freedom, [c] Medium square

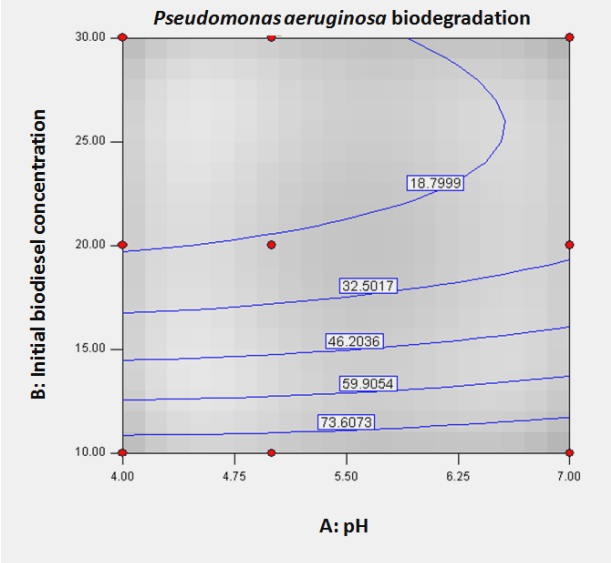

Fig. 2. Contour plot of biodiesel degradation by *Pseudomonas aeruginosa.*

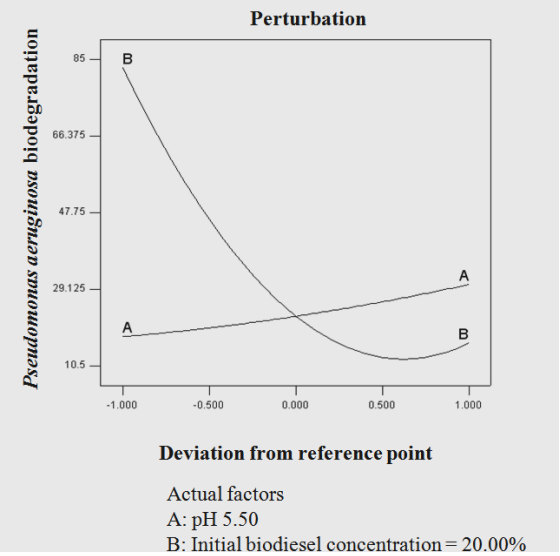

Fig. 3. Perturbation plot of the *Pseudomonas aeruginosa* independent variables: pH(A) and biodiesel initial concentration (B).

The rapid degradation of biodiesel esters is a characteristic that has been identified in other studies (Prince *et al.*, 2008), including those that suggest the use of biodiesel as a co-substrate in the bioremediation of recalcitrant substances (Taylor and Jones, 2001).

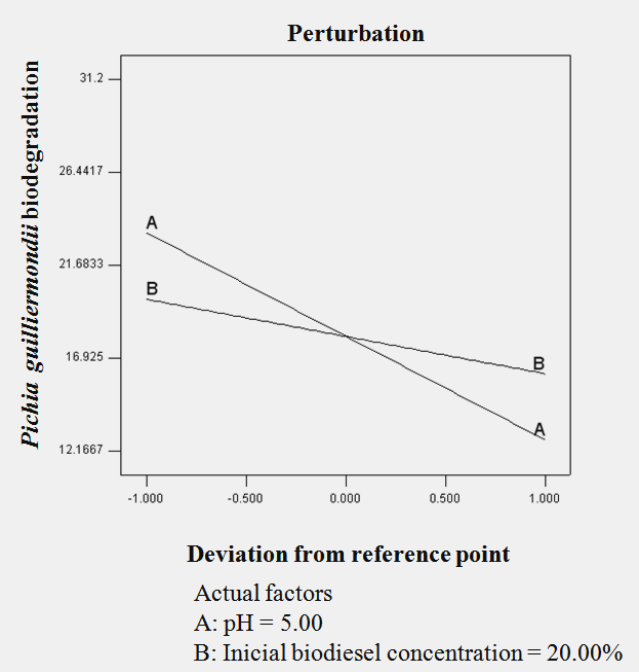

Fig. 4. Perturbation plot of the *Picchia guilliermondii* independent variables: pH(A) and initial concentration (B).

Ranganathan *et al.* (2008) observed a greater uptake of esters with more than sixteen carbons. The same phenomenon was observed in this study, and in the chromatographic analyses (Figs. 5 and 6), we noticed a greater usage in the range from palmitate (16 carbons) to linolenic acids (18 carbons).

Biodiesel biodegradation most likely occurs in the following order: 1) ester cleavage by an esterase to produce a fatty acid and associated alcohol and 2) fatty acid degradation via the Krebs cycle, respiratory metabolism or direct incorporation in cellular lipids. *Pseudomonas aeruginosa*, *Pseudomonas mendocina*, *Bacillus subtilis* and other microorganisms present potential for biodiesel biodegradation (Vieira *et al.*, 2006), which was observed with *Pseudomonas aeruginosa* in the current study, where there was almost total consumption of 10% biodiesel.

It is important to note that the final pH values detected were lower, but in the case of *Picchia guilliermondii*, the acidification of the medium was greater, probably due to an increased formation of acidic metabolic products, which corroborates with previous studies (Madigan *et al.*, 2004).

CONCLUSIONS

The biodegradation capacity of biodiesel varied in relation to the degree of contamination of the medium and the species of microorganism. There was an interaction between the parameters of biodiesel concentration and initial pH value.

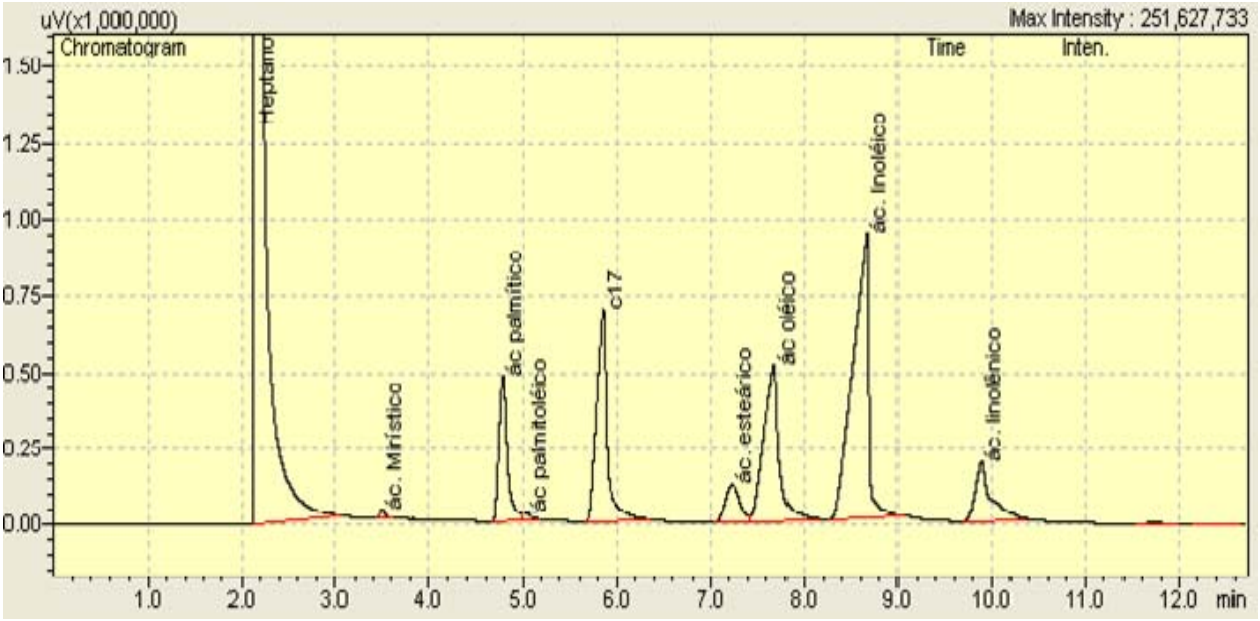

Fig. 5. Cromatogram obtained after actuation of *Pseudomonas aeruginosa* in a medium containing 10% biodiesel and initial pH at 7.0.

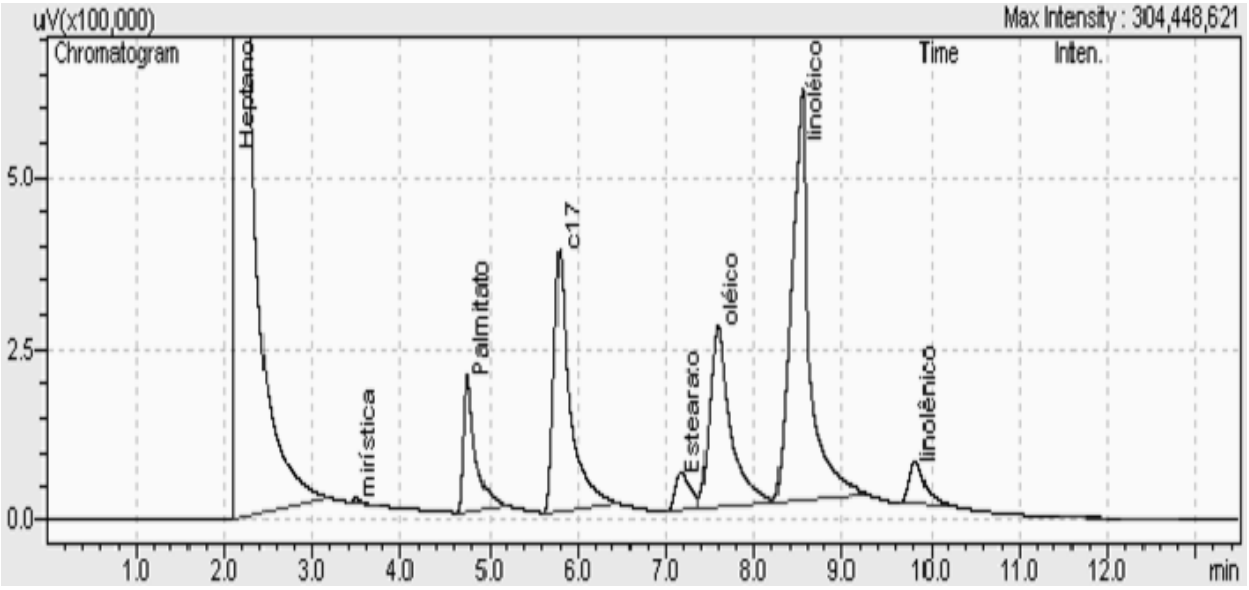

Fig. 6. Cromatogram obtained after actuation of *Picchia guilliermondii* in a medium containing 10% biodiesel and initial pH at 4.0.

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