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Improved Enzyme Stability in Lipase-Catalyzed Synthesis of Fatty Acid Ethyl Ester from Soybean Oil

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Abstract In this work, we describe the optimization of the ethanolysis of soybean oil by the enzyme Lipozyme™ TL-IM in the lipase-catalyzed biodiesel synthesis and the improvement of the enzyme stability over repeated batches. The studied process variables were: reaction temperature, substrate molar ratio, enzyme content, and volume of added water. Fractional factorial design was used to analyze the variables so as to select those with higher influence on the reaction and then perform a central composite design to find the optimal reaction conditions. The optimal conditions found were: temperature, 26 °C; substrate molar ratio, 7.5:1 (ethanol/oil); enzyme content, 25% in relation to oil weight; and added water, 4% in relation to oil weight. Under these conditions, the yield conversion obtained was 69% in 12 h. The enzyme stability assessment in repeated batches was carried out by washing the immobilized enzyme with different solvents (*n*-hexane, water, ethanol, and propanol) after each batch. In the treatment with *n*-hexane, around 80% of the enzyme activity still remains after seven cycles of synthesis, suggesting its economical application on biodiesel production.

Keywords Biodiesel · Lipases · Response surface methodology · Enzyme stability · Organic solvents

Introduction

Biodiesel is a mixture of mono-alkyl esters obtained from vegetable oils extracted from plants such as soybean, jatropha, rapeseed, palm, sunflower, corn, peanut, canola, and cottonseed, among others, and is considered as a carbon neutral fuel emission due to the

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fact that the carbon generated by its burnt in the exhaust of motors is originally fixed from the atmosphere [1]. The reaction can be either catalyzed by inorganic compounds such as acids, alkalis, and salts, or by biological catalysts, the lipases enzymes [2–4].

The use of lipases, which allows for mild reaction conditions and easy recovery of glycerol, largely prevents the drawbacks encountered with chemical catalysis, such as high energy consumption, difficulty in glycerol recovery, and a high amount of alkaline wastewater from the catalysts [5]. Several reports describe lipase-catalyzed alcoholysis reactions in solvents and solvent-free media, especially the reaction parameters affecting the rates of lipase activities in alcoholysis reactions [4, 6–11].

Several aspects will have impact on the enzymatic synthesis of biodiesel, such as enzyme content, molar ratio between reagents oil and alcohol, type of alcohol, use of organic solvent in the reaction, amount of added water in the reaction mixture, reaction temperature, and reuse of the enzyme in repeated operations. The optimization of the reaction parameters involved in lipase-catalyzed biodiesel synthesis is commonly made by varying one factor at a time and keeping the others constant, but this method is costly and inefficient, as it fails to explain relationships between the variables and the response when there is interaction between the variables [4, 8, 12].

Response surface methodology (RSM) is an effective statistical technique for the investigation of complex processes. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results, and it is a faster and less expensive method for gathering research data than the classical method [13]. The use of RSM was reported by some authors for biodiesel enzymatic production [9–11, 14].

Concerning stability over time, enzymes will lose their activity due to a series of factors that must be addressed during enzymatic catalysis. Some of these are the enzyme leakage from supports in which they are attached when in immobility, inhibition by the substrate, thermal inactivation, or the loss of their spatial conformation (change in the active site). Therefore, to reduce biocatalysis cost and make it economically competitive, for an industrial use, compared to chemical catalysis, it is important to extend enzyme activity as long as possible or keep it by the largest number of batch reactions (cycles) possible.

The aim of this work was to optimize the lipase-catalyzed ethanolysis of soybean oil through response surface methodology in a solvent-free system and to improve the stability of the immobilized enzyme in repeated batches. The studied reaction parameters were temperature, substrate molar ratio, enzyme content, and added water to evaluate their effects on yield conversion. The stability of the immobilized lipase during repeated batch runs was investigated by washing the immobilized lipases with some solvents, water, ethanol, propanol, and *n*-hexane.

Material and Methods

Chemicals

A commercial immobilized lipase from *Thermomyces lanuginosus* (Lipozyme TL-IM) was kindly donated by Novozymes™ Latin America (Araucária, Paraná, Brazil) and used in all experiments. Refined soybean oil was purchased in a local market and used without any previous treatment. Ethanol and other chemicals were of analytical grade.

Synthesis and Analysis

Different molar ratios of ethanol were added to 2.75 mmol of soybean oil into 50-ml Erlenmeyer flasks, followed by the addition of different amounts of water and enzyme. The mixtures of soybean oil, ethanol, lipase, and water were stirred in an orbital shaker (200 rpm) at different reaction temperatures for 12 h according to the experimental design. After this time, 5 ml of distilled water was added followed by centrifugation (2,500×g, 15 min, 4 °C). The lower phase containing glycerol was analyzed by high-performance liquid chromatography (HPLC). To verify that the glycerol could be related to the liberation of esters, free fatty acids in the soybean oil and in the product reaction were periodically monitored by tritation with NaOH [15]. This was necessary so as to show that the hydrolysis reaction was not favored instead of transesterification.

Glycerol concentration was determined by HPLC with a refractive index detector (Perkin Elmer Series 200, USA) and a Phenomenex RHM monosaccharide column (300× 7.8 mm), at 80 °C, using ultrapure water as eluent, flow of 0.6 ml min^{-1}, and sample volume of 20 μl. The percentage yield conversion was calculated as follows:

$$\text{Conversion yield} = \left[\frac{\text{mmol glycerol}}{\text{mmol initial soybean oil}}\right] \times 100\,\% \tag{1}$$

Experimental Designs

To determine the optimal conditions for ethanolysis reaction of soybean oil catalyzed by Lipozyme TL-IM, we carried out a fractional factorial design 2^{4-1}, with the independent variables temperature, substrate molar ratio, enzyme content, and added water, varying in two levels and four replications of the central point. Table 1 presents the variables with the respective levels used in the fractional factorial design (FFD).

After selecting the variables with higher influence on transesterification, a central composite design (CCD) was employed to obtain the optimum conditions for biodiesel synthesis. The variables, along with their coded and uncoded values, are given in Table 2. In the CCD, each of the three selected variables were varied at five levels. The design was made up of eight factorial points, six axial points (two axial points on the axis of design variable), and four replications of the central point. In each case, the percentage yield conversion was determined. Second-order polynomial equation for the variables was as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \tag{2}$$

Table 1 Process variables and their levels used in FFD 2^{4-1}.

Variables	Coded levels		
	−1	0	1
Temperature (°C)	20	40	60
Substrate molar ratio (ethanol/soybean oil)	3:1	7.5:1	12:1
Enzyme content (% by oil wt.)	5	15	25
Added water (% by oil wt.)	0	5	10

Table 2 Selected variables and their levels used in CCD.

Variables	Coded levels				
	−1.68	−1	0	1	1.68
Temperature (°C)	20	26	35	44	50
Enzyme content (% by oil wt.)	5	10	17.5	25	30
Added water (% by oil wt.)	0	4	10	16	20

Substrate molar ratio was fixed at 7.5:1 (ethanol/soybean oil)

where Y is the response variable, β_0 the constant, β_i the coefficient for the linear effect, β_{ii} the coefficient for the quadratic effect, β_{ij} the coefficient for the interaction effect, X_i and X_j the coded level of variables x_i and x_j. The above quadratic equation was used to plot surfaces for the variables.

Statistical Analysis

The experimental designs and results analysis were carried out using Statistica 7.0 (Statsoft, USA). The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities, $p(t)$, were determined by Student's t test; the second-order model equation significance was determined by Fisher's F test. The variance explained by the model is given by the multiple determination coefficients, R^2. For each variable, the quadratic models were represented as contour plots (2D).

Enzyme Stability

After the transesterification reaction, the immobilized enzyme was separated from the reaction medium by simple filtration and submitted to different treatments before being reused in a new reaction. The treatments were performed by washing with different solvents. The solvents were *n*-hexane, propanol, ethanol,and water. The enzyme was washed with these solvents and then dried for 24 h at 40 °C. As a control, a parallel experiment was carried out without solvent washing.

Results and Discussion

Fractional Factorial Design

Experimental data and the matrix for the FFD for the optimal conditions for ethanolysis reaction of soybean oil catalyzed by Lipozyme TL-IM are presented in Table 3. FFD consisted of eight factorial points and four replications at the central point. The highest yield of conversion (48.83%) was obtained in treatment 2 (20 °C, 12:1 ethanol/soybean oil, enzyme content 25% and without added water). It was clearly observed that the negative effect of the temperature exerts over the enzyme activity, with the higher temperature (60 °C, treatments 5–8) producing low yields of conversion. In a FFD 2^{4-1}, the main effects can be calculated and used to indicate which variables must be included in the following design as well as to define the new levels of the variables. Table 4 shows the estimated main effects and their p values. Temperature, enzyme content, and added water presented significant

Table 3 Experimental design and results of FFD 2^{4-1}.

Treatment	Temperature	Substrate molar ratio	Enzyme content	Added water	Yield conversion (%)
1	20	3:1	5	0	30.90
2	20	12:1	25	0	48.83
3	20	12:1	5	20	13.57
4	20	3:1	25	20	26.89
5	60	12:1	5	0	4.47
6	60	3:1	25	0	7.47
7	60	3:1	5	20	4.55
8	60	12:1	25	20	4.73
9 (C)	40	7.5:1	15	10	6.49
10 (C)	40	7.5:1	15	10	8.63
11 (C)	40	7.5:1	15	10	6.39
12 (C)	40	7.5:1	15	10	8.32

effects ($p<0.1$) and were selected to be optimized in the CCD, but only the enzyme content positively affected the transesterification reaction, which means that an increase in this variable leads to an increase in yields conversion.

Central Composite Design

Central composite design is used to find the optimal conditions that maximize the yield conversion of the lipase-catalyzed transesterification reaction for biodiesel synthesis. As in the initial screening of the variables, the substrate molar ratio showed no significant effect; this variable has been fixed in its central point (7.5:1, ethanol/soybean oil) for all experiments in CCD. The option for this combination, which is above the stoichiometric molar ratio of 3:1, is justified by the fact that an excess of alcohol ensures higher reaction rates and minimizes diffusion limitations [16]. Moreover, the presence of larger amounts of substrate generally increases the probability of substrate–enzyme collision, leading to an increased yield conversion [13]. Table 5 shows the 18 treatments of the three selected variables and the percentage yield conversion for each experiment.

The higher yield conversions were obtained in treatments 5, 11, and 7, respectively, with conversions of 50% or more. Some authors [10, 11] who showed higher yields of conversion have carried the transesterification out in the presence of organic solvents. Although in some cases the presence of organic solvents in the reaction can favor the conversion, it represents a further step in the biodiesel purification process, thus impacting the costs of its production.

Table 4 Statistical analysis of FFD 2^{4-1}.

Variables	Effect	p value
Temperature[a]	−13.63	0.0253
Substrate molar ratio	−2.54	0.6130
Enzyme content[a]	9.40	0.0916
Added water[a]	−10.25	0.0707

[a] Statistically significant at 90% of confidence level

Table 5 Experimental design and results of CCD.

Treatment	Temperature	Enzyme content	Added water	Yield conversion (%)
1	26	10	4	32.82
2	44	10	4	6.83
3	26	10	16	25.13
4	44	10	16	3.44
5	26	25	4	72.93
6	44	25	4	8.99
7	26	25	16	51.05
8	44	25	16	5.99
9	35	5	10	12.17
10	35	30	10	24.99
11	35	17.5	0	59.46
12	35	17.5	20	22.11
13	20	17.5	10	24.81
14	50	17.5	10	3.03
15 (C)	35	17.5	10	19.74
16 (C)	35	17.5	10	22.73
17 (C)	35	17.5	10	20.12
18 (C)	35	17.5	10	19.19

Model Fitting and ANOVA

The experimental data have been adjusted to the proposed model by the second-order polynomial Eq. 2, and the adequacy of the model was performed by analysis of variance and the parameters R and R^2. The second-order polynomial model is presented in Eq. 3.

$$\begin{aligned} Y = {} & 1.660 + 1.676X_1 - 0.026X_1^2 + 5.521X_2 - 0.007X_2^2 - 6.627X_3 + 0.210X_3^2 \\ & - 0.114X_1X_2 + 0.053X_1X_3 - 0.038X_2X_3 \end{aligned} \tag{3}$$

where Y is the percentage yield conversion, and X_1, X_2, and X_3, are the uncoded values of temperature, enzyme content, and added water, respectively.

Statistical testing of the model was done by the Fisher's statistical test for ANOVA. The computed F value (5.96) was highly significant (p=0.009). The goodness of a model can be checked by the determination coefficient (R^2) and correlation coefficient (R). The determination coefficient (R^2=0.87) implies that the sample variation of 87% for biodiesel production is attributed to the independent variables and can be explained by the model. The closer the value of R (correlation coefficient) is to 1, the better the correlation between the experimental and predicted values. Here, the value of R (0.93) suggests a satisfactory representation of the process model and a good correlation between the experimental results and the theoretical values predicted by the model equation.

Effect of Parameters

Linear, quadratic, and interaction effects for the variables temperature, enzyme content, and added water are presented in Table 6. The variable that presented the highest effects was temperature, producing a negative effect on enzymatic transesterification reaction. This had already been seen in the FFD. Therefore, in the CCD, the range of this variable has been changed to a maximum of 50 °C, restricting the previous condition that was 60 °C.

Table 6 Statistical analysis of CCD.

Variable	Effect	Standard error	p-value
Mean	20.373	0.783	0.0001
Linear			
X_1[a]	−28.472	0.853	<0.0001
X_2[a]	13.590	0.853	0.0005
X_3[a]	−14.490	0.853	0.0004
Quadratic			
X_1X_1[a]	−4.182	0.894	0.0184
X_2X_2	−0.827	0.894	0.4234
X_3X_3[a]	15.161	0.894	0.0004
Interactions			
X_1X_2[a]	−15.330	1.110	0.0008
X_1X_3[a]	5.795	1.110	0.0136
X_2X_3	−3.450	1.110	0.0530

[a] Statistically significant at 95% of confidence level

However, this change had not shown the desired effect, and we observed some thermal denaturation of the enzyme, causing a decrease in the yields of conversion.

The enzyme content shows a positive effect in the transesterification reaction, indicating an increase in the yield conversion with the increase of the amount of enzyme. Because the enzyme has a very high cost, it is important to determine the appropriate amount to obtain high yield conversions, but there is a limit above which the increase in enzyme content will not affect product formation and reaction rate remains constant. Among the limiting factors, low substrate concentration, presence of activators or inhibitors, and mass transfer effects are the most important [13].

Although water does not participate in the alcoholysis reactions, the control of the water content is important for several reasons: water acts as a “lubricant”, maintaining the enzyme in the active conformation; water participates in many mechanisms that cause enzyme inactivation; water promotes the aggregation of particles of enzyme; at high water content, limitations of diffusion of substrate may occur; water can promote the hydrolysis of the substrate, thus decreasing the yield of products [8]. In our work, the amount of added water presented negative effect on the transesterification reaction, which means that the increase in the water content did not favor the synthesis of biodiesel, decreasing the reaction efficiency. The optimum content of water will be, therefore, a combination of minimizing the reaction of hydrolysis and maximizing the enzyme activity for the transesterification reaction [16].

The relationship between reaction variables and response can be better understood by examining the series of contour plots depicted in Figs. 1a–c, which were generated from the predicted model.

Figure 1a shows that increasing enzyme content and lowering temperature will have a positive effect in the yield of reaction. High temperatures showed no influence on the yield conversion even when combined with the increase of enzyme concentration. Figure 1b shows that the combined increase of temperature and added water caused a decrease in the yields of conversion. Finally, results depicted in Fig. 1c show that the increase in the concentration of water has antagonistic effects in the yields of conversion when compared to the concentration of enzyme.

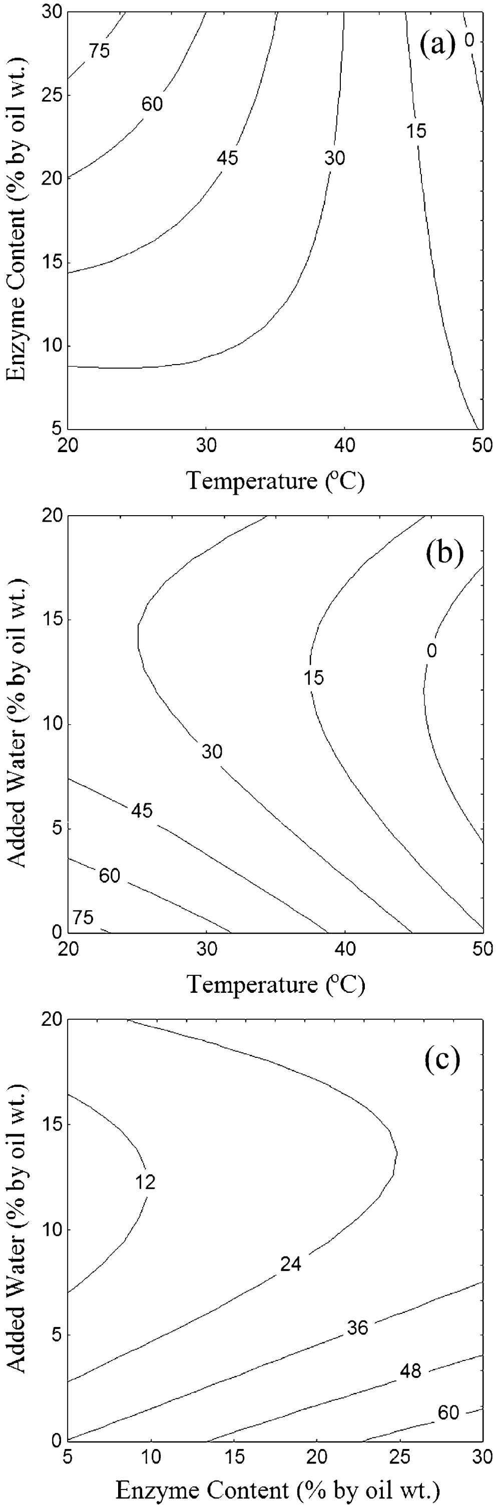

Fig. 1 Contour plots of yield conversions of ethanolysis of soybean oil by Lipozyme TL-IM in a solvent-free system. **a** Temperature vs enzyme content; **b** temperature vs added water; **c** enzyme content vs added water. The *numbers inside the contour plots* indicate yields of conversion (%) at given reaction conditions. In each figure, the missing variable was fixed at the central point

Optimal Conditions and Model Validation

Thus, the optimal conditions for biodiesel synthesis catalyzed by Lipozyme TL-IM were found to be as: temperature 26 °C; enzyme content 25%; added water 4%; and a substrate molar ratio, which had been previously defined, 7.5:1 ethanol/soybean oil. Under these conditions, the theoretical value for the yield of reaction predicted by the model is 66.01%. For the validation of the proposed model, an experiment was conducted under optimized conditions. The test was conducted with four repetitions, and the average yield with the standard deviation obtained was 69.04±1.34%, showing good correlation between the experimental results and the statistical values predicted by the model. Figure 2 presents the time course of lipase-catalyzed biodiesel synthesis. Samples were taken along the time to accompany the ethyl ester synthesis. It was observed that after 12 h, the yield of conversion remains constant.

Enzyme Stability

Immobilized enzyme presents the advantage that it can be reused for several times, but its activity decreases along repeated batches due to many factors such as desorption. Therefore, we tried to improve the stability of Lipozyme TL-IM by washing the immobilized system with different solvents after every batch of synthesis. The experiments were done using the optimal conditions previously obtained. Figure 3 shows the results of the relative yield conversions of treatments with ethanol, propanol, *n*-hexane, water, and the control as percentage of the first batch reaction. For all polar solvents (ethanol, propanol, and water), the enzyme activity remaining after seven batches was approximately of 25%. In the treatment with *n*-hexane, the only non-polar solvent tested, around 80% of the enzyme activity was still maintained in relation to the first batch. During the repeated uses of the lipase, it was also observed that a substrate/product layer gradually formed on the

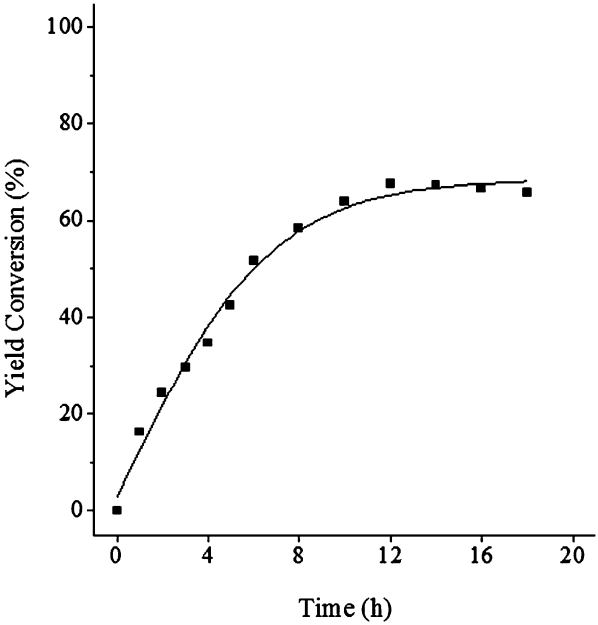

Fig. 2 Time course for lipase-catalyzed biodiesel synthesis at the optimum conditions. Conditions were: temperature 26 °C; enzyme content 25%; added water 4%; and ethanol/soybean oil molar ratio 7.5:1. Experimental points represent the mean of three experiments

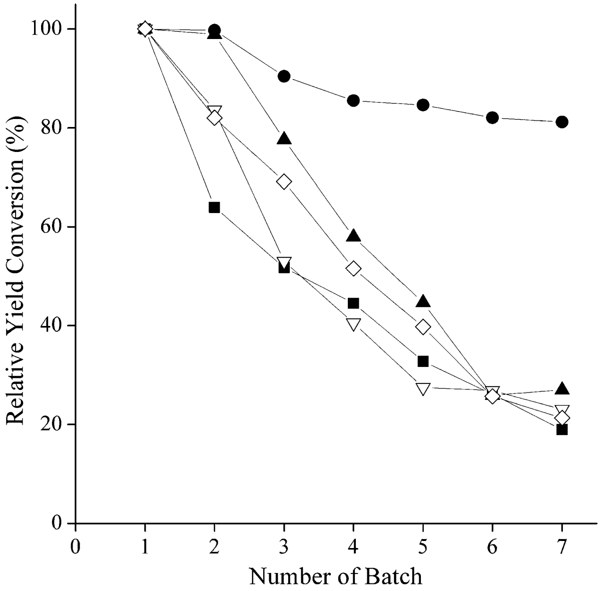

Fig. 3 Stability of Lipozyme TL-IM over repeated batches submitted to different treatments. Control (*filled square*); hexane (*filled circle*); ethanol (*filled triangle*); propanol (*open inverted triangle*); water (*open diamond*)

surface of the enzymatic support, which could cause the loss of activity by limiting substrate and product diffusion [3]. As the main components of the mixture are non-polar (oil/biodiesel), the use of a non-polar solvent to wash the immobilized lipase helps to remove the substrate/product layer formed on the enzyme surface.

Conclusions

The process of biodiesel synthesis can be either achieved by chemical or enzymatic catalysis. While the chemical process has the advantage of being cheaper, it produces highly polluting streams, and several operations are required to avoid environmental contamination and reuse of its by-products. In contrast, enzymatic bioconversion is cleaner but more expensive at present. Therefore, research must be forward to make this technology economically feasible. The optimization of ethanolysis of soybean oil by Lipozyme TL-IM in a solvent-free system through the response surface methodology was successfully obtained in this work, and it is an important step towards process improvement. Under the optimized conditions tested by us, a high yield of conversion of 69% was achieved. Moreover, the comparison between the predicted values by the second-order polynomial model and the values obtained experimentally showed good agreement, showing that the empirical model derived from the RMS can be appropriately used to describe the relationships between the reaction parameters and the response and can, therefore, be used in the enzymatic biodiesel synthesis. Repeated batches of enzyme washes with *n*-hexane showed the high stability of the system, with activities of 80% still remaining after seven cycles. Further research is granted to scale up this model system and to check for its economical feasibility.

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