Study on Synthesis Parameters of Lipase-Catalyzed Hexyl Acetate in Supercritical CO_2 by Response Surface Methodology

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ABSTRACT: Hexyl acetate, a short-chain ester with fruity odor, is a significant green note flavor compound that is widely used in the food industry. The ability of immobilized lipase from *Rhizomucor miehei* (Lipozyme IM-77) to catalyze the transesterification of hexanol with triacetin in supercritical carbon dioxide was investigated in this study. Response surface methodology and a 3-level–3-factor fractional factorial design were adopted to evaluate the effects of synthesis variables, such as reaction time (30 to 90 min), temperature (35 to 55°C), and pressure (1500 to 3500 psi), on percent molar conversion of hexyl acetate. The results showed that reaction time and pressure were the most important parameters and temperature had less effect on percent molar conversion. Based on canonical analysis, optimal synthesis conditions were as follows: reaction time 69.0 min, synthesis temperature 46.7°C, pressure 2640 psi. The predicted value was 75.6% and the actual value was 77.3% molar conversion.

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KEY WORDS: Enzymatic synthesis, hexyl acetate, lipase, optimization, supercritical carbon dioxide.

Hexyl ester (i.e., hexyl acetate) is an extremely aromatic compound with green notes that is widely used in the food industry (1). Traditionally, it has been isolated from natural sources or produced by chemical synthesis. Nowadays, there is a growing demand for natural flavors that are produced by enzymes (2). The present worldwide market for natural "green notes" is estimated to be 5–10 metric tons each year at a current market price of US$ 2,500–6,000/kg. However, with the steadily growing demand for natural flavor compounds that contain green notes, these compounds are in increasingly short supply (3). Therefore, the biosynthesis of such esters by lipase-catalyzed reactions under mild conditions has become of much current commercial interest. In the most favorable conditions, an optimized enzymatic synthesis of hexyl ester improves the conversion yield and reduces the costs of production. This way would benefit the manufacturers and be more appealing to the consumers.

A variety of commercially available lipases and cutinase are known to catalyze ester-synthesis reactions in nearly anhydrous organic solvents and supercritical carbon dioxide ($SCCO_2$) (4). The typical investigations carried out so far in $SCCO_2$ were summarized by Miyawaki and Nakamura (5). The parameters affecting the activities of lipase on the esterification reaction include reaction time, synthesis temperature, pressure, substrate solubility, substrate and enzyme concentrations, added water content, pH memory, and acyl donors (4). The synthesis of geranyl acetate by transesterification catalyzed by immobilized lipase from *Rhizomucor miehei* was studied in $SCCO_2$ and compared with that in *n*-hexane (6). To improve the stability and reactivity of enzyme in $SCCO_2$, cutinase from *Fusarium solani* was immobilized and used to catalyze the esterification of hexanol with hexanoic acid; the results showed that the enzyme lost 10% of its activity over 6 d (7).

Response surface methodology (RSM) is a useful statistical technique for investigating complex synthesis processes, especially for the optimal synthesis of a lipase-catalyzed reaction. Compared with a one-factor-at-a-time design, which is adopted most frequently in the literature, the fractional factorial experimental design was more efficient in reducing the number of experimental runs and time for investigating the optimal conditions of short-chain ester synthesis. RSM has been successfully applied for optimizing conditions in food research and ester production by lipase in organic solvents (8–11), but has not been reported for optimizing the biosynthesis of hexyl ester in $SCCO_2$.

The present work focuses on the reaction parameters that affect *R. miehei* lipase (Lipozyme IM-77)-catalyzed transesterification of hexyl acetate using triacetin as acyl donor in $SCCO_2$. Our purposes were to better understand relationships between the factors (reaction time, temperature, and pressure) and the response (percent molar conversion) and to determine the optimal conditions for hexyl acetate synthesis by means of RSM and canonical analysis.

MATERIALS AND METHODS

Materials. Immobilized lipase (TAG hydrolase, EC 3.1.1.3; Lipozyme IM-77, 7.7 BAUN/g, water 5.4% w/w) from *R. miehei* supported on macroporous weak anionic resin beads was purchased from Novo Nordisk Bioindustrials, Inc. (Bagsvaerd, Denmark). Hexanol, triacetin (99% pure), and tributyrin (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO). Molecular sieve 4Å was purchased from Davison Chemical (Baltimore, MD) and *n*-hexane was obtained from Merck Chemical Co. (Darmstadt, Germany). All other chemicals were of analytical reagent grade.

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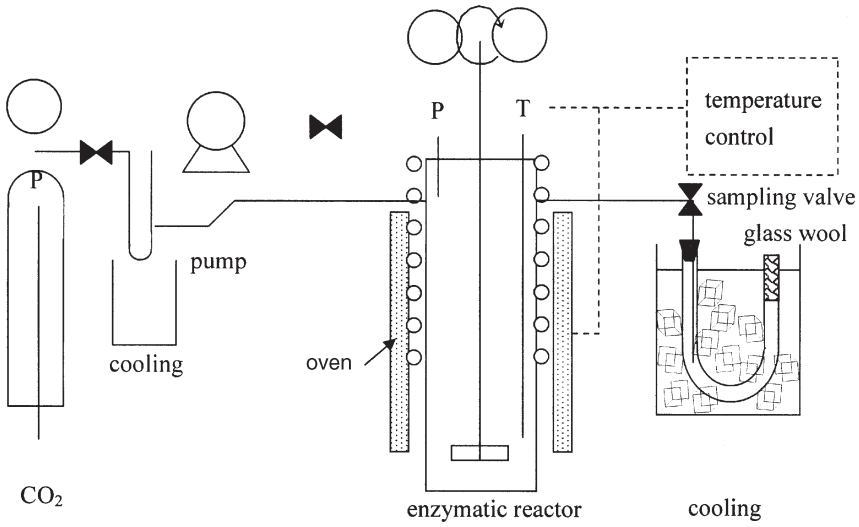

SCHEME 1

Esterification method in $SCCO_2$. All materials were dehydrated by molecular sieve 4Å for 24 h. Hexyl acetate synthesis in $SCCO_2$ was carried out in the apparatus presented in Scheme 1 (where P represents pressure probe and T means temperature probe). The batch bioreactor, with an internal volume 50 mL, was filled with reactants and immobilized lipase. The whole system was placed in a temperature-controlled oven to prevent any possible temperature gradient. According to our previous report (10), the optimal substrate molar ratio (hexanol/triacetin) was around 1:2, and 10% lipase (by weight of hexanol) was enough to reach the yield of 50%. To reduce the number of reaction parameters, the substrate and enzyme levels were fixed in this study. For a typical transesterification reaction, the reaction mixture contained 100 mM hexanol, 200 mM triacetin, and 51.1 mg lipase (10% lipase by weight of hexanol). All reactions were carried out in duplicate.

Extraction and analysis. The enzyme and any residual water were removed by passing reaction media through an anhydrous sodium sulfate column. Before sample analysis, the reactant was mixed with an equal volume of an internal standard solution (50 mM tributyrin). Analysis was then done by injecting a 1-μL aliquot into a Hewlett-Packard 4890 gas chromatograph (Avondale, PA) operated in a splitless mode and equipped with an FID. A DB-5 fused-silica capillary column (30 m × 0.32 mm i.d.; film thickness 1 μm; J&W Scientific, Folsom, CA) was used. Injector and detector temperatures were set at 280 and 300°C, respectively. Oven temperature was maintained at 50°C for 2 min, elevated to 200°C at a rate of 50°C/min, held for 4 min, and then increased to 300°C at a rate of 70°C/min. Nitrogen was used as carrier gas. The percentage yield (percent molar conversion) was defined as (mmol hexyl acetate ÷ mmol initial hexanol) × 100% and was estimated using peak areas integrated by on-line Hewlett-Packard 3365 Series II ChemStation software. One percent molar conversion is equal to one millimole lipase-catalyzed hexyl acetate in this study.

Experimental design. A 3-level–3-factor fractional factorial experimental design with three replicates at the center was employed in this study, requiring 15 experiments (12). The variables and their levels selected for the study of hexyl acetate synthesis were reaction time (30–90 min), temperature (35–55°C), and pressure (1500–3500 psi). The lowest temperature, 35°C, and pressure, 1500 psi, employed in this study were higher than the critical temperature, 31.1°C, and pressure, 1045 psi, at which $SCCO_2$ conditions are reached. Therefore, all the experiments were performed at supercritical conditions in this work. Table 1 shows the independent factors (x_i), levels, and experimental design in terms of coded and uncoded values.

Statistical analysis. The experimental data (Table 1) were analyzed by the response surface regression (RSREG) procedure of SAS software to fit the following second-order polynomial equation (13):

$$Y = \beta_{k0} + \sum_{i=1}^{3} \beta_{ki} x_i + \sum_{i=1}^{3} \beta_{kii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{kij} x_i x_j \qquad [1]$$

where Y is response (% molar conversion); β_{k0}, β_{ki}, β_{kii}, and β_{kij} are constant coefficients; and x_i are the uncoded independent variables. Canonical analysis, one part of the RSREG SAS output, calculates the stationary point, that is, the values of variables at which the first derivatives of the response are zero.

RESULTS AND DISCUSSION

Although hexyl acetate can be synthesized chemically, and the enzymatic synthesis of hexyl acetate in *n*-hexane was good in our previous study (10), $SCCO_2$ has a number of advantages with respect to conventional organic solvents, including high diffusivities and low viscosities that increase mass transfer of the substrate into the catalyst particles, easy solubilization, and easy product recovery without a trace of solvent, the latter being the most attractive aspect, especially in the food industry (6). Therefore, there is a special need to

TABLE 1
Fractional Factorial Design and Observed Experimental Data for 3-Level–3-Factor Response Surface Analysis

Treatment no.[a]	Factors: Reaction time (min) x_1	Factors: Reaction temperature (°C) x_2	Factors: Reaction pressure (psi) x_3	Experimental values (molar conversion) (%)	Predicted values (molar conversion) (%)
1	90 (1)[b]	55 (1)	2500 (0)	65.76	64.21
2	90 (1)	35 (–1)	2500 (0)	53.81	54.31
3	30 (–1)	55 (1)	2500 (0)	41.83	41.32
4	30 (–1)	35 (–1)	2500 (0)	46.28	47.83
5	90 (1)	45 (0)	3500 (1)	48.10	53.43
6	90 (1)	45 (0)	1500 (–1)	44.38	40.08
7	30 (–1)	45 (0)	3500 (1)	30.25	34.54
8	30 (–1)	45 (0)	1500 (–1)	34.94	29.60
9	60 (0)	55 (1)	3500 (1)	60.18	56.39
10	60 (0)	55 (1)	1500 (–1)	44.70	50.55
11	60 (0)	35 (–1)	3500 (1)	63.85	58.01
12	60 (0)	35 (–1)	1500 (–1)	41.76	45.54
13	60 (0)	45 (0)	2500 (0)	72.07	74.32
14	60 (0)	45 (0)	2500 (0)	72.79	74.32
15	60 (0)	45 (0)	2500 (0)	78.10	74.32

[a]The treatments were run in a random order.
[b]The values (–1), (0), and (1) are coded levels.

employ supercritical fluids as solvents during the synthesis of lipase-catalyzed hexyl acetate.

The time course for the transesterification of hexanol with triacetin in $SCCO_2$ by Lipozyme IM-77 is shown in Figure 1A. The molar conversion of hexyl acetate increased up to 80% at 60 min and 70% at 120 min. With respect to temperature, the conversion of hexyl acetate in $SCCO_2$ increased significantly from 35 to 50°C, but it decreased at 55°C (Fig. 1B). Figure 1C shows the effect of varying pressure in experiments conducted for 60 min at 45°C. The conversion significantly increased when pressure was increased from 1500 to 2500 psi, but it slightly decreased as pressure exceeded 2500 psi. The selection of the reaction variable range needs to be extremely precise in the 3-level–3-factor fractional factorial design; otherwise, the optimal condition of synthesis cannot be found inside the experimental region by analyses of statistics and contour plots. Therefore, the variables and their levels selected in this study were: reaction time (30–90 min); temperature (35–55°C); and pressure (1500–3500 psi), as shown in Table 1. Table 1 also shows the actual yields obtained from experiments and the predicted yields derived from the model. Both values were reasonably close, with the sum of residual 0 and average relative deviation 3.44, indicating that the statistical analysis used in this study was practical.

The RSREG procedure for Statistical Analysis System (SAS) (13) was employed to fit the second-order polynomial

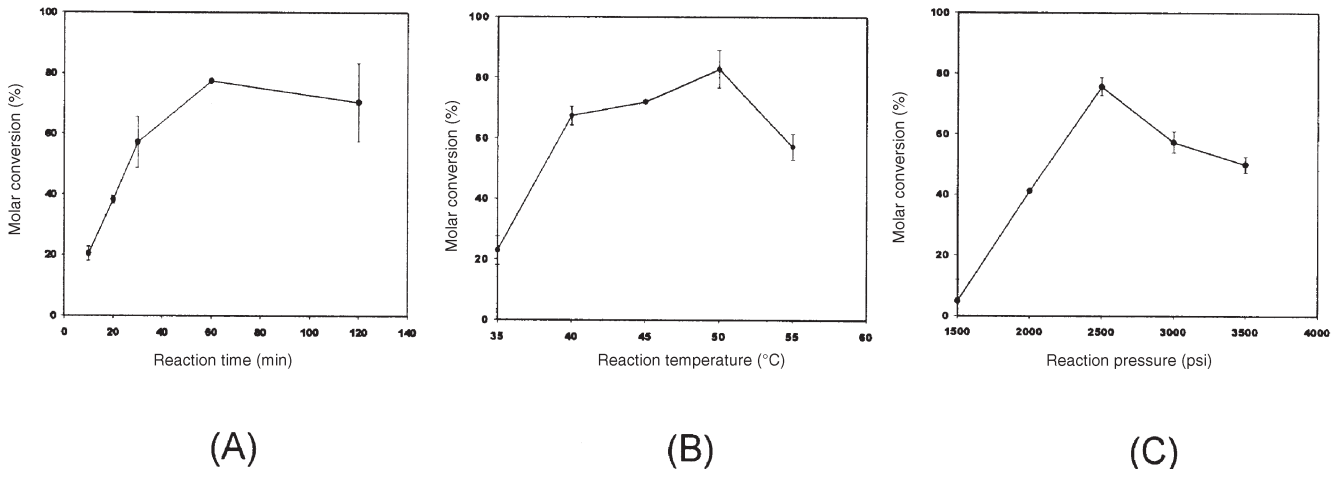

FIG. 1. (A) Time course at 458°C and 2500 psi; (B) effect of temperature at 60 min and 2500 psi; (C) effect of pressure at 60 min and 45°C, for lipase-catalyzed transesterification of hexanol and triacetin in supercritical CO_2 ($SCCO_2$). The reaction was carried out in a 50-mL bioreactor containing 100 mM hexanol, at a substrate molar ratio of 2:1 (triacetin/hexanol), 10% lipase (by wt of hexanol) Lipozyme IM-77 and without added water. 1 psi = 6.8947 kPa. Error bars represent ±1 SD.

TABLE 2
ANOVA for Synthesis Variables Pertaining to the Response Percent Molar Conversion[a]

Source	df	Sum of squares	Prob > *F*
Model	9	2806.060464	0.0216
Linear	3	604.411219	0.0664
Quadratic	3	2105.744148	0.0053
Cross product	3	95.905097	0.5755
Lack of fit	3	196.413115	0.1455
Pure error	2	21.699361	
Total error	5	218.112475	
R^2	0.928		

[a]R^2 is the coefficient of determination.

TABLE 3
ANOVA for Joint Test

Factor	Degrees of freedom	Sum of squares	Prob > *F*[a]
Time (x_1)	4	1686.415	0.0143
Temperature (x_2)	4	40.486	0.5153[b]
Pressure (x_3)	4	1275.653	0.0255

[a]Prob > *F* = level of significance.
[b]Not significant at *P* = 0.05.

Equation 1 to the experimental data, represented as percent molar conversions (Table 1). Among the various treatments, the greatest molar conversion (78.10%) was treatment #15 (60 min, 45°C, 2500 psi), and the smallest (only 30.25%) was #7 (30 min, 45°C, 3500 psi). From the SAS output of RSREG, the second-order polynomial Equation 1 is given below:

$$Y = -197.982 + 1.828x_1 + 3.815x_2 + 0.093x_3 - 0.020{x_1}^2 + 0.014x_1x_2 - 0.046{x_2}^2 + 0.00007x_1x_3 - 0.00017x_2x_3 - 0.00002{x_3}^2 \quad [2]$$

With the very small *p*-value (0.0216) from Table 2 (ANOVA) and a satisfactory coefficient of determination ($R^2 = 0.928$), the second-order polynomial model (Eq. 2) was highly significant and adequate to represent the actual relationship between the response (percent molar conversion) and the significant variables. Furthermore, the overall effect of the five synthesis variables on the percent molar conversion of hexyl acetate was further analyzed by a joint test (Table 3), which revealed that time (x_1) and pressure (x_3) were the important factors, exerting a statistically significant overall effect ($P < 0.1$) on the response, molar conversion of hexyl acetate; temperature (x_2) had a less significant effect ($P > 0.1$) on this reaction. Because the real relationship between response and factors was either unknown or too complex, the simple empirical second-order equation was assumed to be adequate in this study.

The relationships between reaction factors and response can be better understood by examining the series of contour plots generated by holding constant either the reaction time (Fig. 2), synthesis temperature (Fig. 3), or pressure (Fig. 4). Figure 2 shows that the reaction time significantly affected the percent molar conversion (50 to 75%) from 30 to 60 min, but between 60 and 90 min the percent molar conversion decreased (~65%). The reason a greater reaction time decreased the yield was that lipase was inhibited by the accumulation of acetic acid from the release of triacetin. Another reason for the decreased yield could also be that organic acids (e.g., acetic and butyric acids) were acting as competitive inhibitors of lipase during esterification (14). There was no significant effect on lipase activity in $SCCO_2$ when the temperature was increased from 35 to 55°C (Fig. 3). Pressure exhibited the most significant effect on percent molar conversion because the yield at 2500 psi (Fig. 4B) was significantly higher than the others (Figs. 4A and 4C).

Optimal synthesis conditions were suggested by a canonical analysis as described by SAS (13). The stationary point (reaction time 69.0 min, synthesis temperature 46.7°C, pressure 2639.9 psi), that is, the values of variables at which the first derivatives of response were zero, were located exactly in the experimental region with the predicted value of 75.6%. The canonical analysis based on the stationary point resulted in the following equation:

$$Y = 75.570 - 4.251\ {W_1}^2 - 16.362\ {W_2}^2 - 18.886\ {W_3}^2 \quad [3]$$

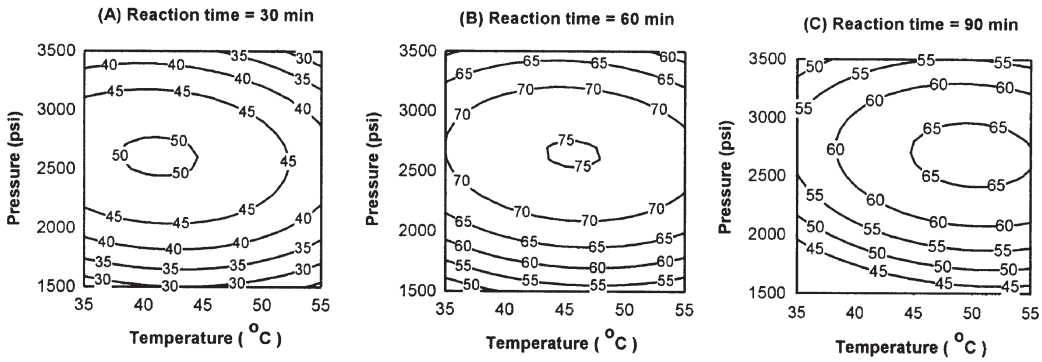

FIG. 2. Contour plots showing response behavior at varying synthesis temperatures and pressures under constant reaction time. The numbers inside the contours represent percent molar conversion to hexyl acetate based on theory at given reaction conditions. The reaction was carried out in a 50-mL bioreactor containing 100 mM hexanol, a substrate molar ratio of 2:1 (triacetin/hexanol), 10% lipase (by wt of hexanol) as Lipozyme IM-77 and without added water.

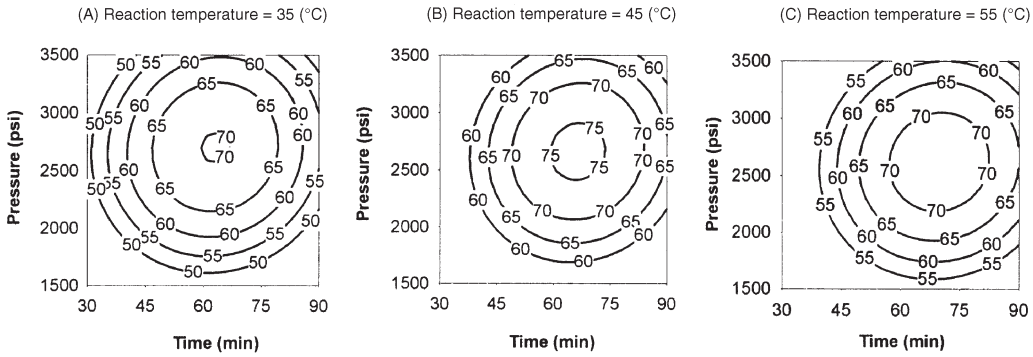

FIG. 3. Contour plots showing response behavior of reaction time and pressure under constant synthesis temperature.

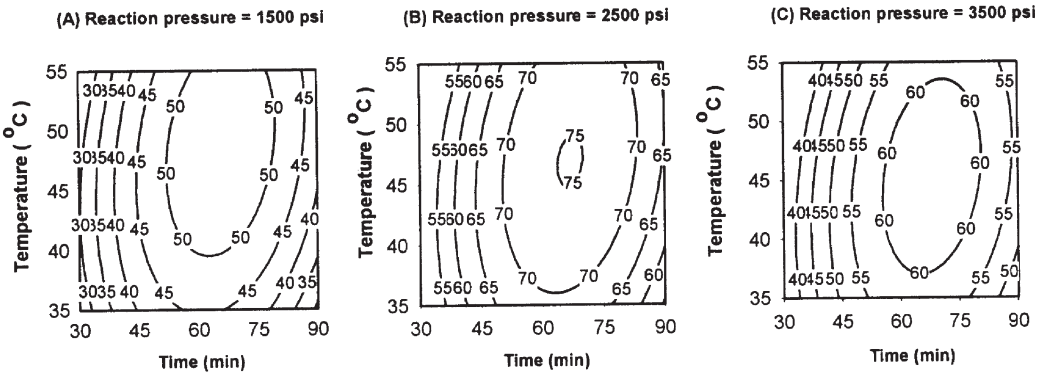

FIG. 4. Contour plots showing response behavior of reaction time and synthesis temperature under constant pressure.

where W_1, W_2, and W_3 are eigenvalues based on coded data and Y is the molar conversion of hexyl acetate (%). All eigenvalues were negative, indicating that the predicted response surface of the stationary point is shaped like a maximum.

The adequacy of the predicted model was examined by additional independent experiments at the suggested optimal synthesis conditions. The predicted value obtained by canonical analysis was 75.6% and the actual value was 77.3 ± 2.5%. A chi-square test (P-value = 0.972, degrees of freedom = 2) indicated that observed values were significantly the same as the predicted values and the generated model adequately predicted the percent molar conversion (15). Thus, the optimization of lipase-catalyzed synthesis for hexyl acetate by Lipozyme IM-77 in $SCCO_2$ was successfully developed by RSM.

Our earlier study (10) showed that the optimal synthesis conditions with 88% molar conversion were as follows: reaction time, 7.7 h; temperature, 52.6°C; enzyme amount, 37.1%; substrate molar ratio, 1:2.7. Compared with our previous work, all the parameters—reaction time (69 min), temperature (47°C), enzyme amount (10%), and substrate molar ratio (1:2)—in this study for hexyl acetate synthesis by lipase in $SCCO_2$ were much enhanced over that in *n*-hexane; however, a slightly lower molar conversion (75%) was obtained. It was concluded that the $SCCO_2$ was a better reaction medium than the classic organic solvent (*n*-hexane) for lipase-catalyzed hexyl acetate biosynthesis by transesterification.

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