SUPPORTING INFORMATION (SI)

Production of liquid biofuel precursors: Optimization and regulation of lipase fermentation and its application in plant oil hydrolysis process

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Media preparation

Seed liquid culture medium: Place 3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone and 10 g/L D-glucose in a 500 mL shake flask and sterilize under high pressure at 116℃ for 25 minutes.

Oblique solid culture medium: Place 3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 10 g/L D-glucose and 20 g/L agar in a large test tube and sterilize under high pressure at 116℃ for 25 minutes.

Fermentation medium: 0.47 g/L $CaSO_4 \cdot 2H_2O$, 9.1 g/L K_2SO_4, 7.5 g/L $MgSO_4 \cdot 7H_2O$, 0.3 g/L $MnSO_4 \cdot H_2O$, 15 g/L triammonium citrate, 20 g/L D-sorbitol, 6.23 g/L KOH, 16.3 g/L H_3PO_4 and 80 g/L soybean oil are placed in a 5 L fermentation tank and sterilize under high pressure at 116℃ for 25 minutes. KOH and H_3PO_4 are first mixed together to prepare a buffer solution to avoid reaction between acid and alkali with other components in the medium.

Trace element solution: Place 30 g/L EDTA Na_2, 3 g/L $ZnSO_4$, 2 g/L $MnCl_2$, 0.6 g/L $CoCl_2$, 0.8 g/L $NaMoO_3$, 6 g/L $FeSO_4$, 9 g/L $CaCl_2$, 0.2 g/L KI, 2 g/L H_3BO_4 and 0.6 g/L $CuSO_4$ in a 50 mL centrifuge tube. Add an appropriate amount of NaOH to adjust the pH to around 5. At this point, it becomes a clear solution and is stored at -20 ℃. Before use, use a filter membrane to remove bacteria.

Vitamin solution: Place 0.1 g/L of biotin, 2 g/L of calcium pantothenate, 2 g/L of vitamin B_1, 2 g/L of niacin, 50 g/L of inositol and 2 g/L of vitamin B_6 in a 50 mL centrifuge tube, store at -20 ℃, and sterilize with a filter membrane before use.

Cultivation methods

First level seed solution: Use an inoculation ring to select a ring of bacterial colonies from the inclined plane and place it in 4 mL of seed solution medium. Shake at 30℃ and 200 rpm for 48 hours.

Second level seed solution: Take 1 mL to 100 mL of the above first level seed liquid culture medium, Shake at 30℃ and 200 rpm for 36 hours.

Olive oil rhodamine B plate culture: Using an inoculation ring, select a ring of bacterial colonies from the inclined plane and place it in 4 mL of seed liquid culture medium. Shake at 30℃ and 200 rpm for 24 hours before taking 20 μL to apply evenly on YPD solid plate and incubate at 30℃ for 48 hours; Inoculate the single colony on the plate to the olive oil rhodamine B plate, and cultivate it in a 30℃ incubator.

Oblique culture: Conduct subculture once every two months, directly use an inoculation ring to select a ring of colonies from the previous generation's slant and transfer it to the slant culture medium. Incubate at room temperature for 48 to 72 hours until obvious colonies can be seen on the slant, and then store it in a 4℃ refrigerator for seed liquid inoculation.

Determination of lipase hydrolase activity

The determination of lipase hydrolysis enzyme activity follows the GB/T 23535-2009 lipase activity detection method, and certain improvements have been made to the determination method for self-made lipases in our laboratory.

(1) Reagent preparation

Substrate solution: Take 800 mL of deionized water, add 20 g of polyvinyl alcohol

(PVA, degree of polymerization 1750 ± 50), heat in a microwave until boiling, stir evenly, and reheat until the solution becomes clear and transparent. Dilute to 1 L with deionized water, filter with gauze, and cool to obtain a 2% polyvinyl alcohol solution. According to the volume ratio of olive oil: 2% polyvinyl alcohol=1:3, the substrate solution can be obtained by treating it with a high-speed homogenizer for 3 minutes (divided into three times, each time treated for 1 minute, and then treated again after cooling). It is now ready for use.

Phosphoric acid buffer solution (pH=8): Weigh 10.71 g of potassium dihydrogen phosphate trihydrate and 0.47 g of potassium dihydrogen phosphate, respectively, and dissolve them in an appropriate amount of water. Dilute to 500 mL with deionized water.

Sodium hydroxide standard solution (0.05 mol/L): accurately weigh 1.000 g of sodium hydroxide particles, dissolve them with 400 mL of deionized water, transfer them to a 500 mL volumetric flask, and fix the volume to the scale.

Phenolphthalein indicator: Weigh 1 g of phenolphthalein and dissolve it in 90% ethanol to a constant volume of 100 mL.

Preparation of enzyme solution to be tested: Take 1-2 mL of lipase sample or fermentation broth, if the sample is in powder form, mash it, and use the phosphate buffer mentioned above to make a volume of 500 mL, which is now ready for use.

(2) Enzyme activity determination: indicator titration method

Add 5 mL of substrate solution and 5 mL of phosphate buffer solution to shake flask (A) and (B), respectively. Add 1 mL of enzyme solution to shake flask (A) and immediately add 20 mL of ethanol to place in a water bath shaker. Add 1 mL of enzyme

solution to shake flask (B) and shake for 10 minutes in a 40 ℃ water bath shaker. Add 20 mL of ethanol and take out together; Add one rotor to each of the (A) and (B) bottles, place it on an electromagnetic stirrer, and titrate with 0.05 mol/L sodium hydroxide solution until the pH is 10.3 and remains constant for 30 seconds. Repeat three times for each lipase sample.

The enzyme activity of lipase preparations is calculated according to the following equation (1):

$$x = \frac{(V_1 - V_2) \times c \times 50 \times n}{0.05} \times \frac{1}{10} \quad (1)$$

Where *x* is the enzyme activity of the lipase sample, U/mL; V_1 is the volume of sodium hydroxide standard solution consumed during the titration of lipase group, mL; V_2 is the volume of sodium hydroxide standard solution consumed during titration of the control group, mL; c is the concentration of sodium hydroxide standard solution, mol/L; "50" is because titrating 1 mL of 0.05 mol/L sodium hydroxide solution is equivalent to consuming 50 fatty acids μmol; n is the dilution ratio of the sample; "0.05" is the concentration conversion of sodium hydroxide standard solution, where 0.05 mol is equal to 50 mmol; 1/10 is the reaction time of 10 minutes, calculated as 1 minute.

Determination of oil in fermentation broth

Separate the remaining soybean oil from the fermentation broth using the methanol chloroform extraction method. Add the mixture to a 50 mL centrifuge tube in the ratio of fermentation broth: methanol: chloroform=1:2:4. Mix well and place in an ultrasonic cleaner for 40 minutes. Dry or rotate to evaporate the underlying solution, repeating each sample three times in parallel. The residual oil content in the

fermentation broth is calculated according to equation (2):

$$\mathrm{m} = \frac{(m_1 - m_2)}{n} \times N \quad (2)$$

Where m_1 is the weight of the centrifuge tube after drying, g; m_2 is the weight of the empty centrifuge tube, g; n is the volume of fermentation broth for extraction, mL; N is the total liquid volume in the fermentation tank, mL.

Analysis method for determining amino acids in fermentation broth by HPLC

(1) The determination of amino acid content requires derivatization, and the derivatization reagents are as follows:

Triethylamine acetonitrile solution: Measure 1.4 mL of triethylamine solution and 8.6 mL of acetonitrile solution, and mix evenly.

Eighteen amino acid standard solutions: Weigh a certain amount of amino acids, dissolve them in 0.1 mol/L hydrochloric acid, and prepare them into 0.5 μmol/mL amino acid standard solution, prepared separately for each amino acid.

0.1 mol/L phenyl isothiocyanate acetonitrile solution: Prepare an acetonitrile solution of 1.25% (v/v) phenyl isothiocyanate.

Sodium acetate solution: Weigh 16.4 g of anhydrous sodium acetate, add 1.8 L of deionized water, adjust the pH to 6.5 with glacial acetic acid, and dilute to 2 L with deionized water to prepare a 0.1 mol/L sodium acetate solution.

(2) Derivatization steps

Firstly, take 2 mL of a mixed standard solution of 18 amino acids and add it to a 15 mL centrifuge tube. Then, add 1 mL of triethylamine acetonitrile solution and 1 mL of 0.1 mol/L phenyl isothiocyanate acetonitrile solution. After thorough mixing, place

the centrifuge tube in a 50 ℃ water bath and react for 45 minutes. After the reaction is completed, take out the centrifuge tube and let it cool. Then add 1 mL of n-hexane, shake well, and let it stand for 30 minutes. At this time, take out the centrifuge tube and separate the lower solution, which can be used for subsequent analysis.

(3) HPLC analysis method

Quantitative analysis of amino acids is performed using a high-performance liquid chromatography (Thermo Scientific Dionex Ultimate 3000) and Sepax AAA column. Mobile phase A is 0.1 mol/L sodium acetate (pH 6.5): acetonitrile=93:7, and mobile phase B is an 80% acetonitrile aqueous solution. The mobile phase program is shown in Table S1.

Table S1 Mobile phase gradient

Time（min）	Mobile phase A（%）	Mobile phase B（%）
0	100	0
0.01	100	0
11	93	7
13.9	88	12
14	85	15
29	66	34
32	30	70
35	0	100
42	0	100
45	100	0
60	100	0

The detection wavelength is λ= 254 nm, flow rate is 1.0 mL/min and column

temperature is 40℃. The derivative sample is filtered through an organic membrane and injected with a sample volume of 2 μL. The peak time of 18 amino acids under the above conditions is shown in Fig. S1. As the liquidity composition changes, the peak time will also change slightly. Each sample will be repeated three times in parallel, mainly using external standard method for quantitative analysis.

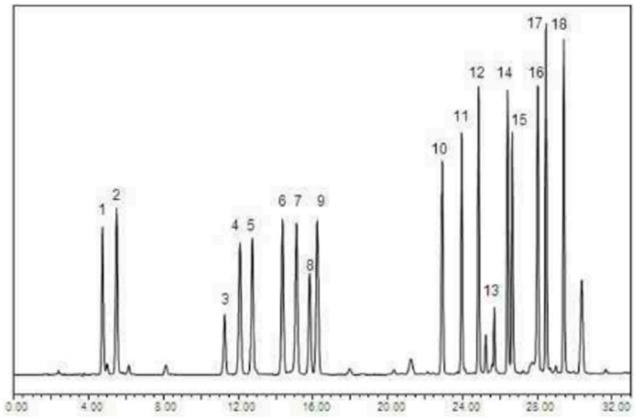

Fig. S1. Sepax AAA detection chromatogram. 1. Asp, aspartic acid; 2. Glu, glutamate; 3. Ser, serine; 4. Gly, glycine; 5. His, histidine; 6. Arg, arginine; 7. Thr, threonine; 8. Ala, alanine; 9. Pro, proline; 10. Tyr, tyrosine; 11. Val, valine; 12. Met, methionine; 13. Cys, cysteine; 14. Ile, isoleucine; 15. Leu, leucine; 16. Phe, phenylalanine; 17. Trp, tryptophan; 18. Lys, lysine.

Table S2. GC data of the reaction procedure under optimum conditions

Time/h	Yield of free fatty acids/%
0	0
1	52.32
2	58.68
3	63.12
4	66.67
5	68.78
6	70.88
7	72.66
8	74.44
9	76.12
10	77.69
11	78.92
12	80.63

Figure S2. Determination of the growth curve of the selected lipase strains to determine the subsequent experimental days.