

Research on Production Method of Sugar Alcohol Based on Enzyme Method Synthesis

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Abstract. The traditional preparation method for carbohydrate fatty acid a, a nonionic biosurfactant, is to use chemical synthesis method. However, the complicated process and low efficient selectivity compels people to search for a more effective method. Enzyme method synthesis of glycolipid is to use lipase to catalyze sugar and acyl donor (e.g. fatty acid or fatty acid ester) in organic solvent to have esterification reaction or exchange reaction. Enzyme is a high efficient and exclusive biocatalyst and can catalyze glycolipid synthesis reaction in non-aqueous media selectively. Its advantages of high efficiency and tenderness have got wide attention. Lipase is mainly for hydrolyzing in aqueous solution and change reaction conditions to make the reaction toward ester synthesis. Therefore, the enzyme catalyzed synthesis is a reversible reaction, the byproduct is water, which determines that the synthesis reaction must be carried out in non-aqueous medium, and water content in the reaction system must be strictly controlled to inhibit reverse reaction. Properties of water content and solvent are important factors influencing the reaction. Other influencing factors include amount of enzyme, selection of acyl donor and receptor and ratio and temperature etc. The article mainly examines the influence of water content in solvent system of tert-amyl alcohol and butanone, influence of fatty acid acyl donors with different number of carbons, and influence of acyl receptors with fructose, glucose and sucrose and the ratio of donor and receptor on the reaction. Experiments show that the optimum synthesis conditions are like this: 0.09g fructose and 0.192g palmitic acid (ratio is 1:1.5) as substrate, 10mL butanone as solvent, 0.1g enzyme, 20 pieces of 4A type molecular sieve (about 0.85g / 10 ml butanone). After 46 hours' reaction, the final balance concentration is 43.6g, fructose conversion rate is 97.5, higher than the same type reaction conversion rate abroad (relatively high fructose conversion rate is 93, and product concentration is less than 30g). Conversion rate of palmitic acid is 88. When the immobilized lipase is repetitively used for 10 times, the enzyme activity merely decreases by 19%.

Keywords: Lipase; Fructose ester; Synthesis; Ultrasound

1. Introduction

Carbohydrate fatty acid ester (abbreviated as glycolipid) usually refers to the ester compounds after esterification between saccharide and aliphatic acid or glycerin. Glycolipid is a substance for energy and substance transfer on cell membrane, widely available in the nature with important physiological activity. In the meanwhile, the substance has amphipathic structure and is an important type of nonionic biosurfactant [1]. Its hydrophilic and oleophilic value (HLB value) can be adjusted by changing the length of fatty acid carbon chain and the number of hydroxy on glycosyl, maximum 1113, minimum 3.54. It can be used either as oil in water type emulsifier or water in oil type emulsifier. It has functions like dirt removal, emulsification, cleansing, dissipation, permeation, diffusion, anti-oxidation, foaming, viscosity adjustment, sterilization, anti-aging, anti-static and so on, mainly used in food, cosmetics, medicine, detergent and fiber industries [2].

Glycolipid is mainly used as surfactant. Though it was discovered 40 years ago, it became commercialized 20 years ago only. And mainly it was produced by chemical synthesis. As sugar has many hydroxies, there must be proper protection since the selectivity of chemical synthesis is not good enough.

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Thus the process was complicated, reaction conditions strict and there were many side products and conversion rate was low. Since the 1980s, especially since 1984, people started to pay attention to enzyme synthesis. Enzyme is a bio-catalyzer with high stereoselectivity, chemical selectivity, high efficiency, and fast and non-toxic catalyzing performance. It can catalyze reaction in non-aqueous media. Therefore, enzyme synthesis has advantages of high efficiency and gentle reaction conditions. It has become a tendency with great potential to use enzyme synthesis.

The enzyme synthesis of glycolipid is a liquid-solid reaction. When liquid passes by solid particles of immobilized enzyme, a relatively static boundary layer will be formed on the surface of the particles, and the reaction compositions must go through this layer to reach the surface of solid. Besides, the reaction speed is not decided by the total substrate concentration or small area substrate concentration but by the concentration of substrate near the enzyme molecule and other conditions of the microenvironment [7]. Therefore, to enhance the substance transfer between substrates and between substrates and enzymes is beneficial for the reaction. Ultrasound, as an effective method for enhancing transfer, is widely studied and applied in many fields. Ultrasound is good for dissolution of low dissolubility solid substrate and for transfer of dissolved substrate to active center of enzyme molecule and timely removal of products, which are quite helpful for reaction. Therefore, it is theoretically feasible to adopt ultrasound to enhance enzyme reaction. The key is to select proper parameters.

2. Selection of Process Conditions for Glycolipid Synthesis by Enzyme Method

Glycolipid synthesis by enzyme method is to use lipase to catalyze carboxyl on fatty acid and hydroxyl on sugar to remove a molecule of water and make them to polymerize to be a glycolipid. The ester exchange reaction is that the carbohydrate replaces the alcohol molecule on original ester to form new ester. On a carbohydrate molecule, there are many hydroxides. Usually, lipase first catalyzes acetylation of primary hydroxyl. On condition that primary hydroxyl is protected, the selectivity of enzyme depends on properties of solvent and aglucon. Enzyme method glycolipid synthesis reaction or ester exchange reaction is influenced by many factors. How to control reaction conditions to get high yield has become the focus of discussion. The main factors include: (1) solvent: different solvents have different dissolving ability to substrate and different inhibitive ability to enzyme activity. The dissolution degree of carbohydrate is great in polar solvents like dimethyl ethyl amide and so on, but there is also strong inhibition to enzyme activity [4]. While in non-polar solvents like normal hexane, acetone and MEK etc., though most activity of enzyme is retained, the solubility of carbohydrate is very low; in TBA and TAA, enzyme can keep activity of 75% and 71%, and carbohydrate also has certain solubility, thus it is widely used. However, they all have certain toxicity, and the content in food additive is strictly controlled. (2) Water content in the system: water is the byproduct in reaction process. The influence of water is complicated. On one hand, it affects balance and lowers conversion rate; on the other hand, enzyme needs certain amount of water to keep catalytic activity and selectivity. Therefore, we have to try to reduce the content of water as much as possible to find optimum content and liveliness on the premise that the enzyme activity and selectivity are kept. (3) Substrate: different acyl receptors and donors have different dissolution degree in solvent, properties of products and yield rate are also different. Different substrate ratios result in different yield rates. In general, to increase yield rate by controlling reaction conditions and optimizing reaction conditions is our target.

The reaction temperature, 60 °C in this experiment, is the active temperature for immobilized lipase Novozym435, at which the activity of enzyme only decreases by 28 after 10 times' continuous usage. At 80 °C, the activity is greatly reduced only after two continuous usages, and after 10 times, it is reduced by more than 90% [5]. As for initial reaction speed, the higher the temperature is, the faster the speed is. The initial reaction speed at 80 °C is more than twice that at 60 °C. Thus the reaction temperature must not be too high. It is reasonable to be at 60 °C-70 °C.

The experiment mainly examines the influence of solvent; substrate and water content on product yield and conversion rate and get the optimum conditions.

Table 1 Experiment instruments and apparatus

Instrument	model no	manufacturer
Water bathing constant temperature vibrator	DSHZ-300	Jiangsu Taicang Laboratory Equipment Factory
Moisture meter	WA-1A	Jiangsu Electric Analysis Instrument Factory
High performance liquid chromatography	426	Alltech
ELSD	2000	Alltech
Analysis scale	TG328B	Shanghai Balance Instrument Factory
Chromatographic column	SP-6800A	Shandong Lunan Ruihong Chemical Instrument Co., Ltd.
Capillary column	AT-SE-30	Lanzhou Chemical and Physical Research Academy
Ultraviolet spectrophotometer	752	Shanghai Precision & Scientific Instrument Co., Ltd.
Rotary evaporator	R201BL	Shanghai Shenke
Vacuum drying box	100A-1	Shanghai Laboratory Instrument Factory

Take all kinds of anhydrous solvents for 10mL each, add in 0.25g (0.139mol) fructose, 0.87ml (0.278mol) oleic acid, about 0.85g 4A molecular sieve, and put them in 60°C (for acetone, it is 50°C) constant temperature vibrator for 30 minutes' balance. Then add in 100mg immobilized lipase Novozyme 435 and vibrate it at 180rpm and measure the initial reaction speed. Stop the reaction after 24 hours and analyze the concentration of the product.

Take anhydrous tert-amyl alcohol and anhydrous butanone for 10ml each, add in 0.25g (0.139mol) fructose, 0.87mL (0.278mol) oleic acid, 0.85g 4A molecular sieve, and put them in 60°C constant temperature vibrator for 30 minutes' balance. Then add in 100mg immobilized lipase Novozyme435 and vibrate it at 180rpm. Take sample for analysis every two hours.

Add water into 10mL anhydrous butanone or anhydrous tert-amyl alcohol and 0.85g 4A molecular sieve. Put it in 60°C constant temperature vibrator and take 10mL sample to test water content periodically.

Take anhydrous butanone as solvent: take 10mL anhydrous butanone and add in 60°C constant temperature vibrator for 10 minutes. Then add in 100mg immobilized lipase Novozyme 435 and vibrate it at 180rpm. Take sample to analyze water content and product concentration every two hours.

Definition of enzyme activity: the amount of enzyme required for producing 1 mol lauric acid normal propyl alcohol ester in one minute under regulated conditions is taken as one enzyme activity unit (PLU). Principle: the immobilized lipase catalyzes lauric acid and normal propyl alcohol to produce lauric acid normal propyl alcohol ester, and the capillary gas chromatography spectrometer measures the yield of lauric acid normal propyl alcohol ester and consumption of lauric acid.

Reaction conditions: 180rps constant temperature water bathing vibrator, 20 minutes' reaction at 60°C, 0.030g enzyme, 0.04mol lauric acid and 0.04mol normal propyl alcohol.

Analysis: after 20 minutes of 60°C constant temperature vibrator reaction, take 5}L clear liquid, add in 995}L normal hexane and use gas chromatography spectrometer or liquid chromatography for analysis.

Observe the reaction speed and final conversion rate taking fructose, glucose and sucrose as acyl receptor, and select the best acyl receptor. When lauric acid is taken as the receptor, take 3 numbers of anhydrous butanone into three 100mL conical flasks, 10mL in each. Add in three types of receptors respectively, each type 0.0005mol, lauric acid 0.001mol, molecular sieve 0.85g, seal the flasks and put them into 60°C constant temperature water bathing vibrator for vibration at 180rpm for 30 minutes. Then add in

100mg immobilized lipase Novozyme 435a. When palmitic acid, oleic acid and stearic acid are used as acyl donor, the process is the same.

Observe the influence on acetylation reaction speed and final conversion rate by capric acid, lauric acid, palmitic acid, stearic acid, oleic acid, oleic acid methyl ester as acyl donor, taking fructose as receptor, and select the best donor. Add in 10mL anhydrous butanone, 0.0005mol fructose, 0.001mol acyl donor, about 0.85g molecular sieve into 100mL conical flask. Seal it and put it into 60°C constant temperature water bathing vibrator to vibrate it at 180rpm for 30 minutes. Then add in 100mg immobilized lipase Novozyme 435 and measure the initial reaction speed and product concentration.

Observe the initial reaction speed, conversion rate and product composition when mole ratio of acyl donor/receptor is 1:1, 1.5:1, 2:1 and 3:1 respectively. Add in 10mL anhydrous butanone, 0.0005mol fructose, 0.0005mol palmitic acid (0.0075mol, 0.001mol, 0.0015mol) and about 0.85g molecular sieve into 100mL conical flask, seal it and put it into 60°C constant temperature water bathing vibrator to vibrate it at 180rpm for 30 minutes. Then add in 100mg immobilized lipase Novozyme 435 and measure the initial reaction speed, product concentration and conversion rate.

Observe the change of initial reaction speed with continuous usage of immobilized lipase Novozyme 435 at 60°C and 70°C and measure the stability of the enzyme. Add in 10mL anhydrous butanone, 0.0008mol fructose, 0.0075mol stearic acid, and about 0.85g molecular sieve, seal it and put it into constant temperature water bathing vibrator (60°C and 70°C respectively) and vibrate it at 180rpm for 30 minutes. Add in 100mg immobilized lipase Novozyme 435 to start reaction and measure the initial reaction speed. Use thin layer chromatography to monitor the reaction process. When it is balanced (the reflection is that the color of fructose at TLC obviously becomes light as there is no solid fructose in the system and the consumed fructose does not get makeup and thus the concentration decreases), filter the immobilized lipase out, clean it with anhydrous butanone and immediately put into the next batch preheated raw material and start the next cycle reaction.

Take fructose palmitic acid as example. Add in solvent anhydrous butanone 30mL, fructose 0.008mol, palmitic acid 0.01mol and about 0.85g molecular sieve. Seal it and put it into 60°C constant temperature water bathing vibrator to vibrate it at 180rpm for 30 minutes. Add in 300mg immobilized lipase Novozyme 435 and react for 30h. Remove the undissolved like molecular sieve and immobilized lipase etc. by filtration. When the liquid becomes concentrated, use silica gel layer chromatographic column. Diameter of silica gel column is 2.5cm, height 40cm. Ethyl acetate: methyl alcohol:water=12:0.5:0.1 (volume ratio), elution speed 2.0mL/min. Collect elution liquid 10mL and carry out TLC monitoring. Combine the elution liquids with the same composition, use rotary evaporator to dry the solvent at 40°C, remove the product to watch glass, dry it in vacuum drying box at ambient temperature (0°C-25°C) to constant weight.

It is used to analyze the water content in samples.

Add in solvent, substrate and molecular sieve as per certain ratio, preheat it for half an hour, add in catalyzer and start timing. Take samples after 10, 20 and 30 minutes and analyze the product concentration. Draw the curve of product concentration and reaction time (h) and get a straight line. The gradient is the initial reaction speed rate.

Reduce pressure and distill it after reaction to dry the solvent. Add in certain amount of DMF to dissolve all substrates and products, analyze the residue amount of substrates and calculate the conversion rate.

Use tert-amyl alcohol as solvent and adopt reduced pressure distillation method to remove by-product water can result in high conversion rate, while when using molecular sieve to remove water, though the initial reaction speed is high, the conversion rate is far lower than ketone solvent. Hereinafter, we will have detailed discussion on the synthesis reaction taking tert-amyl alcohol and butanone as solvent. The curve of product concentration along with time is shown in figure 1 during the synthesis reaction using immobilized lipase Novozyme 435 to catalyze fructose and oleic acid. The product concentration in tert-amyl alcohol reaches the peak at 6h and then greatly reduces and reaches balance at 12h. The total ester concentration at balance is 27.0. In butanone, the total product concentration reaches 82.9 at 33h. The initial reaction speed of ester synthesis in tert-amyl alcohol is 2.6 times that in butanone. This reflects the advantage of tert-amyl alcohol with high dissolution ability of fructose. At the beginning of reaction, the water content in the

pretreated system by molecular sieve is very low. With the same water content, a high fructose concentration means strong reaction force, and then a high initial reaction speed can be achieved.

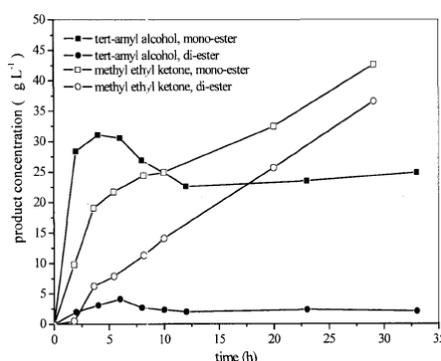


Fig. 1: curve of concentration of reaction product in different solvents

3. Conclusion

Molecular sieve dehydration method is adopted to search for and optimize glycolipid synthesis conditions. It is found that when this method is adopted, butanone is the best solvent. Water in butanone is easy to be removed by molecular sieve method. Use the same amount of molecular sieve to control the water content in medium during reaction can make the water content in butanone lower than that in tert-amyl alcohol by 40. The optimum amount of molecular sieve is 0.85g/10mL solvent. When different acyl receptors are used as reaction substrates, there is large difference in initial speed and product concentration because of different structures and dissolution degrees. Comparatively, fructose is the most proper substrate as it has high dissolution degree in butanone. The best donor is palmitic acid. When the acyl donor/receptor ratio is 1.5, the conversion rate of two substrates is high, respectively 88% and 97.5. To increase the ratio can increase the reaction speed and product concentration, but the conversion rate of acyl donor will decrease and result in waste of raw material. Temperature has big influence on enzyme activity and the reaction. The higher the temperature is, the faster the reaction speed is. However, high temperature is not good for stability of enzyme. At 60°C, Novozyme 435 has good stability in butanone; at 70°C, the stability greatly reduces. The loss of enzyme activity reaches 60 when it is repetitively used for 10 times. Thus the proper temperature is 60°C. The preliminary synthesis conditions are found out: substrate 0.09g fructose, 0.192g palmitic acid (ratio 1:1.5), solvent 10ml butanone, 0.1g enzyme, about 0.85g 4A molecular sieve.

4. References

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