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Scientific Research

Directional Breeding of High Itaconic Acid Yielding Strain of *Aspergillus terreus* with a New Plate Technique

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ABSTRACT

Itaconic acid is commercially produced by the cultivation of *Aspergillus terreus* using starch hydrolysate as carbon source. The degree of hydrolysis had a great influence on itaconic acid production which was suitable when corn starch was saccharified at 35 DE. The *α*-amylase was sufficient to drive the starch hydrolysis to the degree. The agar plate assay with LiCl treatment provided a rapid, simple and unequivocal method for screening large numbers of colonies for itaconic acid producing strains. It was learned by experience that the strains on the plates with thick hyphae and light-colored spores often accompanied high itaconic acid production. A strain, designated Ast165, producing itaconic acid with a high yield, was successfully obtained by directional breeding of metabolic end products resistant strains. The itaconic acid concentration produced by Ast165 was 53.8 g/l from 100 g/l of starch hydrolysate in shake flasks. The conversion rate was 61.3%, which was the highest value found in tests.

Keywords: *Aspergillus Terreus*; Itaconic Acid; Corn Starch; Dextrose Equivalent; Enzymatic Hydrolysis

1. Introduction

With a new interest in sustainable development, the chemical industry is making many attempts to replace petrochemical-based monomers with natural ones. Itaconic acid (IA), or methylene succinic acid, is an unsaturated acid with conjugated double bonds and two carboxyl groups (**Figure 1**). IA and its ester are used for the synthesis of fiber, resin, plastic, rubber, paints, surfactant and lubricant [1-3]. IA was discovered by Baup as a thermal decomposition product of citric acid at 175˚C [4]. This was the most primitive preparation method of IA. But chemical synthesis processes can not really compete with fermentation by fungi because the cost remains high [5]. The biosynthesis by fungi from carbohydrates was first reported by Kinoshita [6], who isolated IA from the growth medium of an osmophilic fungi, *Aspergillus itaconicus*. Later, other fungal strains, mainly of the species *Aspergillus terreus*, were found to be more suitable [7]. New biotechnological methodologies involving fermentation processes and technologies that use alternative cheap substrates as the carbon source are currently under investigation and development [8]. The favorite substrates for IA production by the *A. terreus* are hydrolysates of starchy materials [9-11]. Corn starch is one of the best carbon sources, since it is very pure, inexpensive, and stable in a mass supply [12]. However, corn starch is not a popular fermentation raw material because it is very difficult to sterilize, due to gelatinization upon heating. The problem of gelatinization of corn starch upon heat sterilization was solved by hydrolyzing the starch using acid or enzymes. Acid hydrolysis of starch required the use of corrosion resistant materials as vessel, gave rise to high colour and salt ash content (after neutralisation), needed more energy for heating and was relatively difficult to control. Therefore, only the starch hydrolysate prepared with enzymes was used as carbon source for IA production in experiments. Strain improvement is another method to reduce the cost of IA production. To date

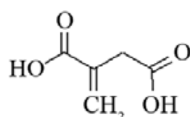

Figure 1. Chemical structure of itaconic acid.

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very little research has been directed at the improvement of itaconic acid production. The screening of an excellent strain will be a promising choice to get the successful results [13].

2. Materials and Methods

2.1. Microorganism and Media

A. terreus T730 was obtained from Laboratory of Food and Fermentation Research, Guangxi University, China.

The seed medium contained (g/l): sucrose, 60; NH_4NO_3, 4; $MgSO_4 \cdot 7H_2O$, 2.5; corn steep liquor, 3; $CuSO_4 \cdot 5H_2O$, 0.006 and pH 3.0. The production medium consisted of (g/l): starch hydrolysate, 100; NH_4NO_3, 3; $MgSO_4 \cdot 7H_2O$, 4; corn steep liquor, 2; KH_2PO_4 0.2 and pH 3.0. The indicator medium consisted of (g/l): starch hydrolysate, 70; NH_4NO_3, 3; $MgSO_4 \cdot 7H_2O$, 4; corn steep liquor, 2; KH_2PO_4, 0.2; Bromocresol green, 0.1 and pH 3.0. The selective medium consisted of (g/l): starch hydrolysate, 70; NH_4NO_3, 3; $MgSO_4 \cdot 7H_2O$, 4; corn steep liquor, 2; KH_2PO_4, 0.2; LiCl, 10; Bromocresol green, 0.1 and pH 3.0.

2.2. Culture Conditions

The experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml of medium. A spore suspension of 10^8/ml was incubated at 35˚C for 48 h on a rotary shaker at 200 rpm. Then 10% of the seed medium was transferred into a 250 ml Erlenmeyer flask containing 45 ml of production medium. The strain cultivation was performed at 35˚C for 120 h on a rotary shaker at 220 rpm. Samples were taken for analysis of pH, total sugar, reducing sugar and IA concentrations.

2.3. Fermentation Substrate

Corn starch used was a commercial grade manufactured by Global Bio-chem Technology Group, China. Corn starch was hydrolyzed using enzymes.

2.4. Enzymatic Hydrolysis of Starch for Fermentation

Enzymatic hydrolysis was carried out by the two-step hydrolysis of corn starch: liquefaction and saccharification. The corn starch and distilled water were mixed to the concentration of 200 g/l with stirrer. The slurry was blended with 0.5% (v/w dry starch) of α-amylase. The liquefaction was performed at 90˚C, pH 6 in a stirred reactor. The termination of liquefaction was determined by the colour of reaction of resultant in enzymatic hydrolysis of starch with iodine chemical. The downstream process was undertaken by following the general processes of saccharification. According to the catalyzing characteristics of glucoamylase, saccharification was carried out at 60˚C, pH 4.3. Dextrose equivalent (DE) was determinated in process of starch hydrolysis. Corn starch hydrolysate at different dextrose equivalent (DE) were used as carbon sources for IA production by *A. terreus*.

2.5. Screening of Strains with High Itaconic Acid Yield

Primary screening: The obtained rearrangement strains with good mycelia growth and spore-production abilities were successively transferred and cultured. Then their spore suspension were transferred onto the selective medium agar plate cultured at 30˚C for 4 d. Single colonies growing fast on the plates with a larger color change zone diameter were selected as the high-output strains through primary screening. Other colonies were discarded. The surface structures of *A. terreus* on the plates were revealed using scanning electron microscopic technique.

Secondary screening: The strains obtained by primary screening were activated in agar slope culture according to the seed medium at 30˚C for 4 d, then transferred into 50 ml of the production liquid medium in a 250 ml flask and cultured at 35˚C for 120 h. At the end of fermentation, the supernatant fluid was used for determinations of total sugar, reducing sugar and IA concentrations.

2.6. The Directional Breeding of Metabolic End Products Resistant Strains

2 g/l itaconic acid was added to the production liquid medium. The spore suspension was transferred into 50 ml of the mixed medium in a 250 ml flask and cultured at 35˚C for 48 h. The fermented liquor was spread on the indicator medium agar plates for screening of strains.

2.7. Analytical Methods

The pH value of culture broth was measured by a pH meter (Model UB-10, DENVER). For the total sugar, the phenol-sulfuric acid method was used [14]. The DNS method was used for the reducing sugar measurement [15]. IA concentration was measured by the bromine absorption method [16]. "Dextrose equivalent" refers to the industry standard for measuring the concentration of total reducing sugars, calculated as glucose on a dry weight basis [17]. Conversion rate is defined as: conversion rate = IA production/(initial medium total sugar content-residual sugar content) × 100%.

2.8. FTIR Spectroscopic Measurement

The fermented liquor was centrifuged. The supernatant was decolorized with actived carbon, and filtered. The

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filtrate was passed through ion exchange resin beds, then concentrated and dried to produce white powder. The powder was examined by Fourier transform infrared (FTIR) spectroscopy, on Nicolet Avatar 370 using a KBr pellet.

3. Results and Discussion

3.1. IA Production Based on the Enzyme Hydrolysate

Enzyme hydrolyzed corn starch was used as carbon source for IA production by fermentation. **Figure 2** showed the effect of degree of starch hydrolysis using α-amylase and glucoamylase on IA production. *A. terreus* can not make good use of raw starch for fermentation production of IA. Soluble solids suspensions of corn starch were assayed during the enzyme action. When DE value of starch hydrolysate as carbon source was 7 in the fermentation medium, the IA concentration after fermentation was 20.0 g/l. The highest IA concentration of 35.2 g/l was the hydrolyzed starch at 35 DE as carbon source. Although DE value of starch hydrolysate continued to increase with the duration of hydrolysis, the IA concentration gradually decreased. It meant that α-amylase and glucoamylase for industry were crude. Some by-products inhibitory to cell growth were produced with time of hydrolysis increasing. To reduce the production of the inhibitory compounds, the degree of enzyme hydrolysis was controlled to 35 DE. The α-amylase hydrolyzes the long starch polymers to shorter chains consisting of five to ten glucose molecules, called dextrins. Unlike starch, these dextrins are water soluble. Then glucoamylase is added to begin the saccharification step. During saccharification, the glucoamylase further hydrolyzes the dextrins down to individual glucose molecules. The α-amylase was sufficient to drive the starch hydrolysis to 35 DE. *A. terreus* cells could utilize the oligosaccharides [9]. As a result, the DE value of starch hydrolysate as carbon source in later fermentation medium was 35.

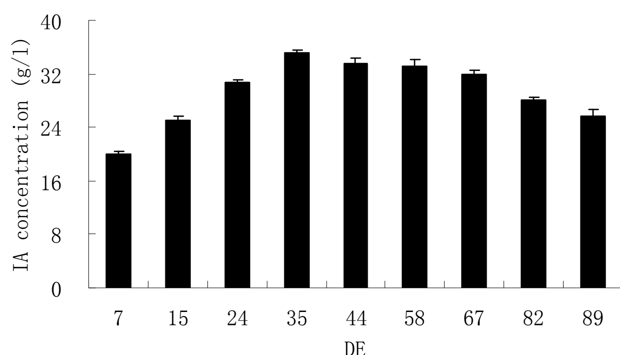
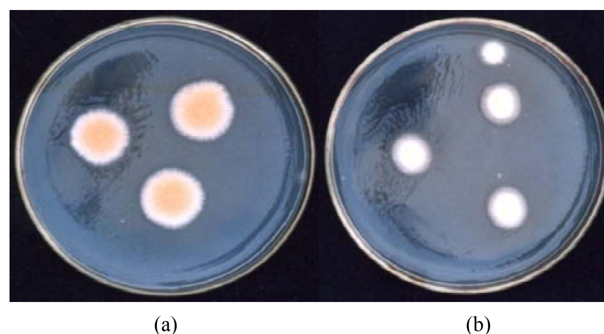


Figure 2. Effect of degree of starch enzyme hydrolysis on itaconic acid production. Results were mean values of three samples, and error bars represented standard deviations.

3.2. Observation of Surface Structure of *A. terreus*

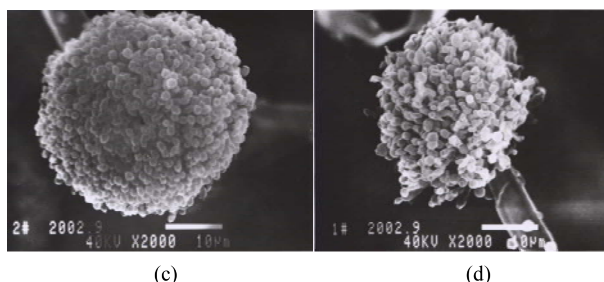
After 2 d incubation at 30˚C, a round, white, cottony mass of hyphae grew out of the indicator medium agar plate. Growth proceeded laterally and in 4 d the colony center became yellow with spore germination as shown in **Figure 3(a)**. In contrast, the strains on the selective medium agar plate were observed in **Figure 3(b)**. After cultured at 30˚C for 4 d, the colonies were comparatively small and the spores were greyish white. It indicated that LiCl in medium inhibited the growth of *A. terreus*.

Scanning electron micrographs of *A. terreus* showed some characteristics of the spores (**Figure 4**) and the hyphae (**Figure 5**). The scanning electron microscope magnified the object 2000 times. *A. terreus* on the selective medium agar plates had smaller conidia heads, less spores and thicker hyphae than *A. terreus* on the indicator medium plates. The observation revealed that LiCl treatment retarded spore germination and yet the hyphae grew strong. A positive correlation existed between LiCl resistance and IA production. It was concluded that *A. terreus* with thicker hyphae had a higher apparent metabolizability and the spores germination rate signified the speed of growth and reproduction of *A. terreus*. The above conclusion has subsequently been verified by numerous experiments. The efficiency of strain selection is



(a) (b)

Figure 3. Colony shape on agar plate. (a) *A. terreus* on the indicator medium plate; (b) *A. terreus* on the selective medium plate.



(c) (d)

Figure 4. Scanning electron micrographs of *A. terreus* spores (×2000). (c) *A. terreus* conidia on the indicator medium plate; (d) *A. terreus* conidia on the selective medium plate.

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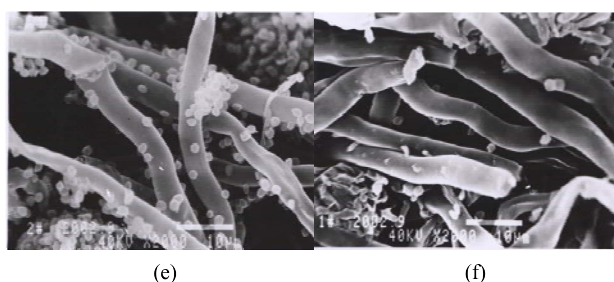
greatly raised, and the intensity of labor is decreased.

3.3. Fermentation in Shake Flasks by Directional Breeding

Single colonies on the indicator plates with a larger color change zone diameter, thick hyphae, light-colored and many spores were selected for shake flask fermentation. 7 strains with high IA production were obtained from 600 single colonies. **Table 1** showed the results of fermentation in shake flasks after directional breeding procedures. The IA concentration in the fermented liquor of parental strain on starch hydrolysate was 35.2 g/l. When the strains were picked up in the screening, IA production after fermentation had been greatly improved. The fermentation medium containing IA could reduce some IA-intolerant strains. The high producing mutant was obtained with strong aconitase activity and anti-feedback inhibition. The strain Ast352 gave the highest IA concentration of 54.5 g/l after fermentation. The highest conversion rate of 61.3% from starch hydrolysate to IA was obtained by strain Ast165 with IA concentration of 53.8 g/l, respectively.

Strain Ast352 and strain Ast165 were serially transferred through agar slant culture. **Table 2** indicated effect of transfer of culture on IA production. A downward trend in the IA production of strain Ast352 had accelerated from the 4th transfer. However, the IA production of strain Ast165 was stable relatively. Simultaneous observation of plate culture showed that the spores of strain Ast352 enhanced the yellow pigment productin and the hyphae were shortened. It demonstrated that strain Ast352 was in degradation. Relative to the other strains, the IA production of strain Ast165 was high and stable; meanwhile, the conversion rate from starch hydrolysate to IA was the highest.



(e) (f)

Figure 5. Scanning electron micrographs of *A. terreus* hyphae (×2000). (e) *A. terreus* hyphae on the indicator medium plate; (f) *A. terreus* hyphae on the selective medium plate.

Table 1. Results of fermentation in shake flasks by directional breeding.

Strain	IA concentration (g/l)	Conversion rate (%)
Ast037	48.6 ± 0.5	56.1 ± 0.6
Ast165	53.8 ± 0.7	61.3 ± 0.6
Ast219	49.3 ± 0.7	57.1 ± 0.6
Ast283	48.3 ± 0.3	55.8 ± 0.7
Ast352	54.5 ± 0.6	59.3 ± 0.4
Ast461	52.1 ± 0.4	59.1 ± 0.5
Ast528	51.7 ± 0.8	57.9 ± 0.3

*Data were the means of triplicate experiments with standard deviations.

Table 2. Effect of transfer of culture on itaconic acid production.

Strain	IA concentratio (g/l)				
	Generation 1	Generation 2	Generation 3	Generation 4	Generation 5
Ast165	53.8 ± 0.6	53.6 ± 0.4	53.2 ± 0.8	52.6 ± 0.7	51.9 ± 0.3
Ast352	54.5 ± 0.5	53.7 ± 0.7	52.1 ± 0.6	50.3 ± 0.4	47.7 ± 0.8

*Data were the means of triplicate determination from flask culture experiments with standard deviations.

3.4. Characterization of IA by FTIR

The purified sample was characterized by the functional groups using FTIR technique. The IR spectrum was presented in **Figure 6**. The sample O-H stretch appears as a very broad band in the region 3100 - 2900 cm^{-1}, centered at about 3000 cm^{-1}. An intense band at 1702 cm^{-1} is associated with the carbonyl C = O stretch of a carboxylic acid. It indicates that the sample is conjugated unsaturated carboxylic acid. The carboxylic acid C-O stretch appears at 1215 cm^{-1}, and the O - H bend is at 1440 cm^{-1} and 910 cm^{-1}. The band at approximately 1635 cm^{-1} is the C = C stretch. The results show that structure of sample is a carboxylic acid with conjugated double bonds, which is consistent with the structure of IA.

4. Conclusions

The results of the present study had indicated the possibility of utilizing the low DE starch hydrolysate as a raw material for IA production. *A. terreus* had the ability to produce IA by using partially hydrolyzed corn starch as carbon sources. Enzymatically hydrolyzed corn starch was used as substrates for IA production by *A. terreus* T730. The degree of hydrolysis had a great influence on IA production which was suitable when corn starch was saccharified at 35 DE. The α-amylase was sufficient to drive the starch hydrolysis to 35 DE.

The agar plate assay with LiCl treatment provided a rapid, simple and unequivocal method for screening large numbers of colonies for IA producing strains. It was learned by experience that the strains on the plates with

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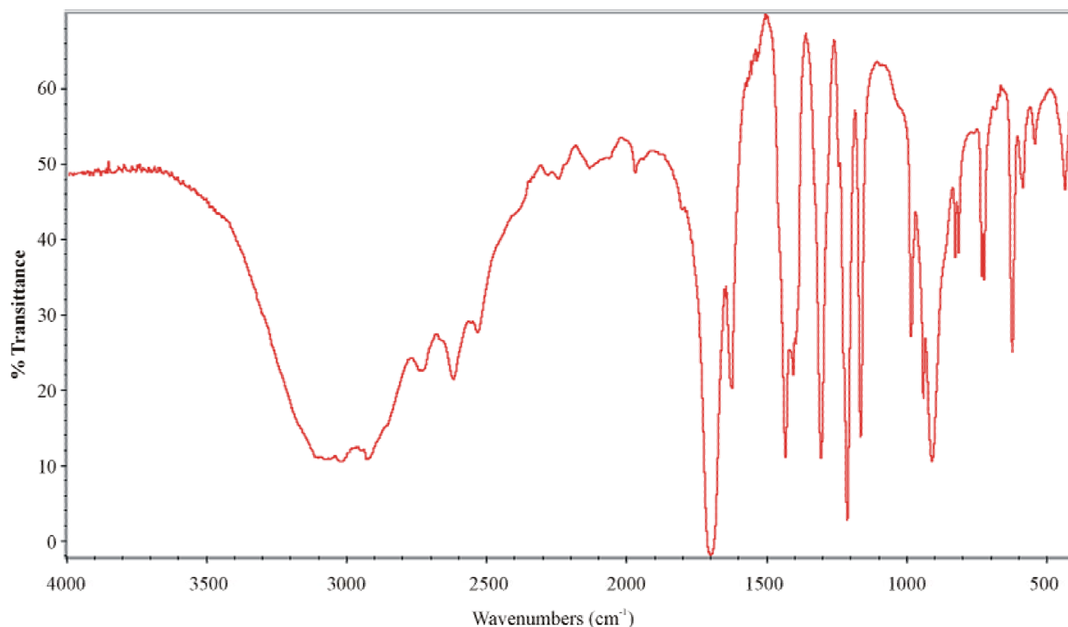

Figure 6. Infrared spectrum of itaconic acid.

thick hyphae and light-colored spores often accompanied high IA production. From the results observed, the directional breeding method developed in the present study can be advocated as more rapid, sensitive, easily visible and reproducible than existing methods, and hence it will certainly be helpful in the rapid screening and isolation of IA producing strain. A strain, designated Ast165, producing itaconic acid with a high yield was successfully obtained by the screening with directional breeding of metabolic end products resistant strains. The production medium consisted of (g/l): starch hydrolysate, 100; NH_4NO_3, 3; $MgSO_4·7H_2O$, 4; corn steep liquor, 2; KH_2PO_4, 0.2 and pH 3.0. The DE value of starch hydrolysate in medium was 35. The fermentation was performed at 35˚C for 120 h on a rotary shaker at 220 rpm. The IA concentration produced by Ast165 was 53.8 g/l using starch hydrolysate in shake flasks, which was 52.8% higher than that of the parental strain. The conversion rate was 61.3%, which was the highest value found in tests.

5. Acknowledgements

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