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Effects of Soya Fatty Acids on Cassava Ethanol Fermentation

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Abstract Ethanol tolerance is a key trait of microbes in bioethanol production. Previous studies have shown that soya flour contributed to the increase of ethanol tolerance of yeast cells. In this paper, the mechanism of this ethanol tolerance improvement was investigated in cassava ethanol fermentation supplemented with soya flour or defatted soya flour, respectively. Experiment results showed that ethanol tolerance of cells from soya flour supplemented medium increased by 4–6% (*v*/*v*) than the control with defatted soya flour. Microscopic observation found that soya flour can retain the cell shape while dramatic elongations of cells were observed with the defatted soya flour supplemented medium. Unsaturated fatty acids (UFAs) compositions of cell membrane were analyzed and the UFAs amounts increased significantly in all tested strains grown in soya flour supplemented medium. Growth study also showed that soya flour stimulated the cell growth rate by approximately tenfolds at 72-h fermentation. All these results suggested that soya fatty acids play an important role to protect yeast cells from ethanol stress during fermentation process.

Keywords Soy fatty acids · Soya flour · Cassava · Ethanol · Tolerance

Introduction

In the past years, biofuels from different renewable resources have received high attention due to the increasing petroleum shortage. Replacement of fossil fuel with biofuels provides great promise for the future. To get the cost-effective energy, starting materials cheaper than edible corn are needed. Recently, cassava is being used widely for ethanol fermentation. It is a tropical root crop and embodies high cassava starch. Since cassava is tolerant to drought, poor

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soils, and has a wide adaptability to different climates, it has been widely planted in the world and regarded as an important inexpensive fermentable source [1–3].

In order to save water resources and to protect the environment, wastewater is usually centrifuged and then the supernatant is recycled to mix with raw material in the cassava ethanol plant. However, high concentrated inorganic salts will be accumulated during circulations and then cause an increase of the osmotic pressure, which has a negative effect on yeast cells survival. The ethanol tolerance of yeast cells falls in this high osmotic stress environment, which will cause the decrease of the final ethanol yield.

In this condition, both of the ethanol tolerance and osmotic resistance were thought to be two important characteristics which affect the ethanol fermentation. Recently, cell membranes have received extensive attention as primary barrier against ethanol and osmotic stress. Many reports have indicated a corelationship between the fatty acid compositions of lipid membranes and ethanol stress tolerance [4, 5].

Membrane lipids are widely recognized as modulators of membrane fluidity and are considered to play an essential role in the tolerance of *Saccharomyces cerevisiae* to ethanol [6, 7]. The membrane fluidity will increase in the presence of ethanol, and the incorporation of unsaturated fatty acid (UFA) into cellular membrane lipids can effectively affect a compensatory decrease in membrane fluidity and lower the membrane leakage [8].

During the fermentation, cell growth and ethanol production are inhibited eventually in the result of gradually increasing ethanol concentration and osmotic pressure. Under severe conditions, the presence of soy flour can boost the fermentation rate, the sugar consumption, and the final ethanol concentration in the medium [9–11]. In this study, the effects of soy flour on the ethanol tolerance, changes in terms of morphology, and fatty acid compositions of yeast cells are investigated. Fermentation wastewater was replaced by distillers dried with solubles (DDS) in the experiment, which made the experiment carried out more conveniently and exactly.

Material and Methods

Material

The cassava (starch content ≈ 73% of dry weight), soya flour, and defatted soya flour were purchased from market. High-temperature α-amylase (Novozymes, 2×10^4 units) and β-glucosidase enzyme (Novozymes, 1×10^5 units) were used for this study.

Distillers dried with solubles preparation was as following: Fermentation solids (lees) were centrifuged at 5,000 rpm for 10 min. The supernatant was collected and concentrated until the solid content is about 40% (how it is transformed from 40% to 96.2%). Then, DDS (solid content=96.2%) was dehydrated after drying at 95°C for 30 min.

Strains

The yeast strains employed in this study were *S. cerevisiae* AYC75, CICC1347, and CICC1625. CICC1347 and CICC1625 were purchased from China Center of Industry Culture Collection. AYC75 was from our collection.

Media and Growth Conditions

The seed culture medium is composed of 10% hydrolyzed cassava sugar and 0.5% yeast extracts. The fermentation medium contained 24% hydrolyzed cassava sugar, 0.15% yeast extracts, and

0.2% ammonia sulfate. Batch fermentation was performed at 30°C with 100 ml of fermentation medium in a 250-ml Erlenmeyer flask with 10% inoculums. To mimic wastewater back condition, cassava DDS (about 40 g per liter) was added to the fermentation medium. Ethanol and sugar concentration were periodically determined. At 12-h intervals, samples were collected and the concentrations of ethanol, residual sugar, and biomass were analyzed, respectively.

Ethanol Tolerance Test

The yeast strains were inoculated in cassava hydrolyzed media containing 12%, 14%, 16%, 18%, and 20% (*v*/*v*) ethanol, respectively. The cultures were incubated at 30°C for 24 h. A Durham tube was used to indicate the ethanol-tolerance properties of the strains. Carbon dioxide gas produced in the Durham tube was expressed as "+".

Preparation of Cell Membrane

The yeast cells were harvested, washed twice with distilled water, and then twice with 1.5 M sorbitol solution. Cells were converted to spheroplasts essentially. Spheroplasts were then spun down by low speed centrifugation (2,000 rpm) and washed twice with 1.2 M sorbitol solution. The total membrane fraction was obtained as a pellet after osmotic lysis of the spheroplasts in 0.017 M NaCl and centrifuged 30,000 rpm for 20 min at 4°C. Then, the plasma membrane fraction was further isolated according to the method of Schibeci et al. [12].

Fatty Acid Extraction

The membrane fractions were dissolved into 1 M potassium hydroxide and ethanol solution by stirring at 80–90°C for 1 h. After saponification completed, the solution was dried in a rotary evaporator to remove ethanol. The soap collected was immediately dissolved in 10 ml distilled water and then extracted twice by 10 ml ether. The pH of the water phase was adjusted to 2.0–3.0 with hydrochloric acid. At this pH, the fatty acid could dissociate from the soap. The solution was extracted twice with 10 ml ether, washed by water for neuter, and then dried by anhydrous sodium sulfate for several hours. After the ether was vaporized, the mixed fatty acids were obtained. The mixture was heated at 60°C for 30 min with 5 ml 4% hydrochloric acid–methanol solution. Three milliliters hexane and 3 ml distilled water were mixed with the fatty acid methyl esters. The solution was washed with distilled water to neuter and dried by anhydrous sodium sulfate.

Fatty Acid Determination

The fatty acid methyl esters were analyzed with gas chromatography–mass chromatography (Varian 4000MS GC-MS, USA). The samples were separated on a VF-5 ms column (30 m×0.25 mm×0.25 μm) using programmed temperature: started at 120°C holding for 4 min then to 280°C at a rate of 5°C/min, holding for 10 min. The transmission line and the trap temperature were 280°C and 220°C, respectively. The carrier gas (Helium 99.999%) was kept at a flow rate of 1 ml/min. For MS analysis, ionization energy was applied and the ionization temperature was 200°C.

Other Methods

Ethanol concentration was determined with gas chromatography as described by Castrillo and Ugalde [13]. Collected samples were decomposed first by addition of 3 M HCl at the boiling

point for 30 min. Total sugars were tested using the phenosulfuric acid method [14], and the glucose is used as equivalent.

Results and Discussion

Effects of DDS on the Cassava Ethanol Fermentation

To measure the ethanol productions, *S. cerevisiae* AYC75, CICC1347, and CICC1625 were incubated in cassava medium, respectively. Whenever needed, 4% DDS (*w*/*v*) was added to the medium. The results were shown in Table 1. Samples with DDS have less carbon dioxide weight losses than the controls without DDS, suggesting that the addition of DDS in cassava can reduce fermentation rate.

Cassava DDS contains some inorganic ions, such as Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, and Fe^{2}, and they increased the osmotic stress of media. The osmotic pressure caused by DDS exerted a strong inhibition on yeast cell growth and ethanol production (Table 1). Under high osmotic pressure conditions, the starch utilization rate of CICC1347, CICC1625, and AYC75 decreased from 93.2%, 92.7%, and 93.0% to 90.5%, 88.4%, and 91.9%, respectively. And ethanol concentration of each strain was 10.3% (*v*/*v*) for AYC75, 9.5% (*v*/*v*) for CICC 1347, and 8.3% (*v*/*v*) for CICC162. These results showed high osmotic pressure caused by DDS extended the fermentation time and decreased the starch utilization rate.

The fatty acids compositions of *S. cerevisiae* cell membrane were determined by gas mass chromatography (Table 2).The major fraction proportion of unsaturated fatty acids of yeast were palmitoleic acid $C_{16:1}$, oleic acid $C_{18:1}$, and linoleic acid $C_{18:2}$. In the membrane sample of AYC75, the palmitoleic acid and the oleic acid were not found and the content of linoleic acid was only 5.81%. However, with the supplement of DDS to fermentation, more UFA was detected and the total content of the UFA increased by 50.0%. In the membrane of CICC1347, oleic acid was not found in the control, while the content of main UFA increased by 47.6% with the addition of DDS.

Effect of Soya Fatty Acids in the Medium on the Ethanol Tolerance of Yeast Cells

Two soy preparations, soya flour (lipids content 19.2%), and defatted soy flour (lipids content 1.1%) were chosen as additives. Yeast AYC75 was grown in cassava medium with different concentration of ethanol supplied with soya flour or defatted soy flour, respectively. The ethanol tolerance of yeast cell with defatted soya flour had no significant changes. In the medium supplied with soya flour, the ethanol concentration yeast could tolerate, increased by as high as 6% (*v*/*v*) compared with that of the control (Table 3).

The addition of soya flour is responsible for the increase of the starch utilization rate and the final achieved ethanol concentration, whereas defatted soya flour had no stimulative function for ethanol fermentation. These results indicated that the lipids in soya flour had active effects. Soya flour may enhance the fermentation rate, the amount of sugar consumed, and the final ethanol concentration in the media [9, 11]. High ethanol concentration through soya flour supplementation was previously studied by several investigators [10] and soya flour was reported to be a good nutrient supplement. Viegas et al. [15] observed a significant increase in glucose consumption and a high final concentration of ethanol when 2% or 4% (*w*/*v*) soya flour was added to the fermentation media. These studies showed clearly that soya flour lead to enhanced fermentation parameters even when it is added to inoculum medium [11] or in the case of immobilized cells [10].

Table 1 Fermentation capability of *S. cerevisiae* in the medium with DDS

Medium	Yeast strains	Weight loss of CO_2 (g)					Ethanol concentration (%, *v*/*v*)	Residual total sugar (g/100 ml)	Starch utilization rate (%)
		12 h	24 h	48 h	60 h	72 h			
		Mean±SD							
Control	CICC 1347	12.5±0.1	22.7±0.3	28.6±0.6	30.5±0.3	30.5±0.3	13.2±0.1	1.16±0.04	93.2±1.1
	CICC 1625	11.3±0.2	20.6±0.2	26.3±0.2	28.4±0.1	28.4±0.1	11.8±0.4	1.72±0.01	92.7±0.8
	AYC75	12±0.1	21.3±0.1	27.9±0.4	30.1±0.5	30.1±0.5	13.1±0.1	1.32±0.03	93.0±1.4
Added with DDS	CICC 1347	9.3±0.2	15.5±0.3	19.6±0.3	22.1±0.3	23.8±0.1	9.5±0.2	1.94±0.04	90.5±1.1
	CICC 1625	6.7±0.2	13.1±0.3	17.3±0.2	20.6±0.1	21.8±0.3	8.3±0.5	2.82±0.05	88.4±0.2
	AYC75	7.5±0.1	15.4±0.2	20.9±0.3	24.7±0.2	26.3±0.1	10.3±0.2	2.93±0.03	91.9±1.0

The values are means of three independent experiments

SD Standard deviation

Table 2 Fatty acid composition analysis of *S. cerevisiae* in presence and absence of DDS

Medium	Yeast strain	Fatty acid composition (%)									Unsaturation index (Δ/mol)
		10:0	10:1	12:0	14:0	16:0	16:1	18:0	18:1	18:2	
		Mean±SD									
Control	CICC1347	8.91±0.19	1.78±0.02	10.02±0.44	3.45±0.21	59.24±1.54	3.85±0.39	8.40±0.57	ND	4.34±0.06	0.14
	CICC1625	2.36±0.01	1.08±0.04	1.84±0.11	1.26±0.08	21.82±1.03	8.76±0.59	5.80±0.37	25.17±1.06	31.91±2.06	0.98
	AYC75	6.94±0.09	ND	8.78±0.32	4.88±0.17	63.84±1.38	ND	9.74±0.10	ND	5.81±0.02	0.12
Added with DDS	CICC1347	8.06±0.31	4.16±0.13	3.92±0.20	2.63±0.15	21.6±0.09	6.09±0.51	12.27±0.09	21.32±0.96	19.95±1.01	0.71
	CICC1625	10.00±0.26	2.06±0.21	4.92±0.07	2.12±0.22	39.98±1.47	7.57±0.31	11.08±0.07	10.94±0.57	11.32±0.53	0.43
	AYC75	4.46±0.20	0.96±0.06	2.22±0.06	1.36±0.11	30.08±2.18	9.99±0.63	5.06±0.32	18.02±1.63	27.85±1.30	0.85

The values are means of three independent experiments

SD Standard deviation, *ND* not detected

Table 3 Effects of soya fatty acids on ethanol tolerance of *S. cerevisiae* in cassava medium with defatted soya flour or soya flour

Medium	Ethanol concentration (%, *v*/*v*)					
	16	18	20	22	24	26
Control	++	+	−	−	−	−
With defatted soya flour	++	++	−	−	−	−
With soya flour	++	++	++	+	+	−

++ Full fermentation, + fermentation, − no fermentation

Effect of Soya Fatty Acids on Ethanol Production in the Media Supplied with DDS

The effect of soya flour and defatted soya flour on ethanol production was examined (Table 4). The final ethanol concentration increased from 11.7% to 14.7% (*v*/*v*) when 3% (*w*/*v*) soya flour was added to the cassava ethanol fermentation by *S. cerevisiae* AYC75. Likewise, the starch utilization rate also reached to 92.5%, improved by 1.2% than control. The final total residual sugar concentration decreased from 2.81 g/100 ml in the control to 1.86 g/100 ml in sample supplied with soya flour. In contrast, the addition of defatted soya flour caused little changes in ethanol concentration, residual total sugar, and starch utilization rate.

Effect of Soya Fatty Acids on the Growth of Yeast Cells

The effects of soya fatty acids on morphology were examined using microscopy (Fig. 1). At the early stage, yeast cells were round or elliptical (Fig. 1a). After 72 h fermentation, micrograph showed yeast cells of the sample with no addition became elongated (Fig. 1b), similar to cells of the control culture with defatted soya fatty acids(Fig. 1c). In contrast, the cells of culture with soya flour maintained normal morphology of yeast (Fig. 1d). Moreover, the number of viable cells experienced a remarkable drop beyond 60 h through 72 h fermentation process (Table 5). At the end of fermentation, the number of viable cells added with soya flour still kept nearly 1×10^8, while the number of control sample and sample treated with defatted soya flour decreased to $0.1–0.2\times10^8$. This may due to the absence of nutrition, the inhibition of ethanol, and the effect of osmotic stress.

Effect of Soya Fatty Acids on the Fatty Acid Composition of Yeast Cell Membrane

S. cerevisiae AMC75 was grown in cassava media supplemented with DDS. The amount of lipids of cell membrane was measured and the results were shown in the Table 6. The UFAs content of yeast cells cultured in media with defatted soya flour had an increase of 0.94%, whereas the UFAs content of cells grown in media supplemented with soya flour increased 4.90%. It is notable that the relative percent content of long-chain fatty acids increased by 1.97% in soya flour medium, while the content increased by only 0.16% when defatted fatty acids was added to the medium. These results suggesting that supplement of soya flour stimulated the synthesis of UFAs in yeast cells. The yeast strain with more UFAs had higher ethanol tolerance and was better suitable for high osmotic pressure fermentation. Similar trends in UFAs composition as a function to stabilize the membranes against osmotic pressure and to increase the efficiency of biochemical reactions have been detected in *Escherichia coli* [16].

Table 4 Effects of soya fatty acids on ethanol production in cassava medium with defatted soya flour or soya flour

Medium	Weight loss of CO_2(g)					Ethanol concentration (%, *v*/*v*)	Residual total sugar (g/100 ml)	Starch utilization rate (%)
	12 h	24 h	48 h	60 h	72 h			
	Mean±SD							
Control	7.4±0.27	15.3±0.41	20.9±1.27	24.4±1.39	26.4±0.49	10.2±0.59	2.81±0.02	91.2±1.63
With defatted soya flour	7.3±0.19	15.2±0.62	21.1±0.94	24.8±0.51	26.6±0.73	10.7±0.28	2.74±0.06	91.4±1.38
With soya flour	8.0±0.10	15.9±0.64	22.1±0.53	26.6±2.03	28.4±2.03	11.7±0.26	1.86±0.07	92.5±0.26

The values are means of three independent experiments

SD Standard deviation

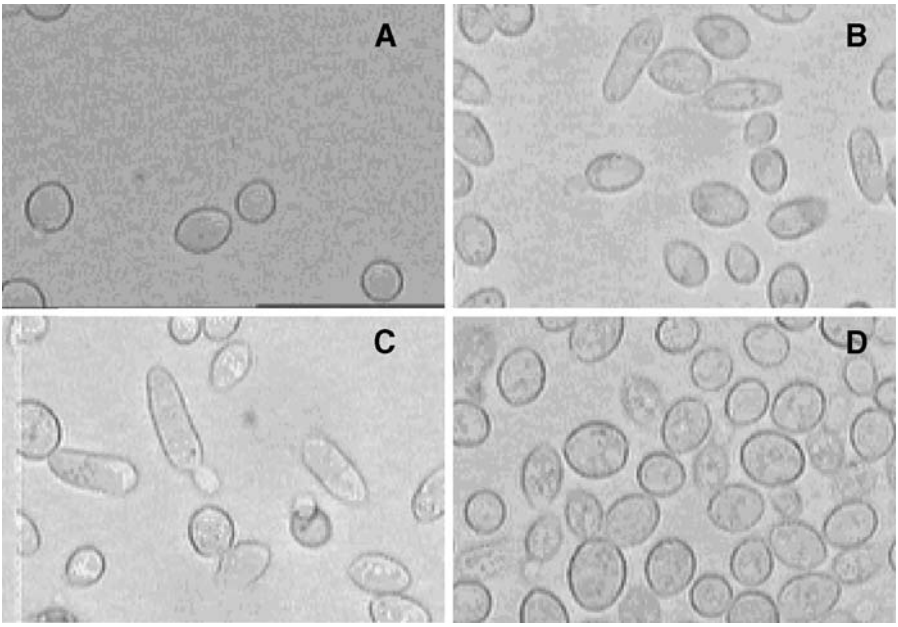

Fig. 1 Effects of soya fatty acids on yeast morphology (magnification, ×400). **a** Early stage of fermentation; **b** at 72 h fermentation without additives; **c** at 72 h fermentation with the addition of defatted soya flour; **d** at 72 h fermentation in the presence of soya flour

Conclusions

In the early stage of fermentation, yeast cells were affected by DDS under osmotic pressure, while in the late stage of fermentation, yeast cells were affected by dual affects: DDS and increased ethanol concentration. We proposed that supplementation of soya flour in the medium led to high viability of yeast, which is consistent with Viegas et al.'s observation [15], while defatted soya flour had no effects. Thus, it can be concluded that soya fatty acids could maintain the cell activity and protect yeast cells from the damages caused by ethanol and osmotic stress.

Table 5 Effects of soya fatty acids on the viability of the yeast cells in the presence of defatted soya flour or soya flour

Medium	The number of viable cells				
	12 h	24 h	48 h	60 h	72 h
Control	2.31×10^8	2.24×10^8	2.42×10^8	2.07×10^8	1.31×10^7
With defatted soya flour	2.19×10^8	2.42×10^8	2.14×10^8	2.21×10^8	2.03×10^7
With soya flour	1.93×10^8	2.12×10^8	2.45×10^8	2.20×10^8	9.46×10^7

Samples were taken during the progressing of fermentation to determine the cell viability as described in "Material and Methods"

Table 6 Effects of soya fatty acids on the fatty acid composition of yeast cell membrane

Medium	Fatty acid composition (%)									Unsaturation index (Δ/mol)
	10:0	10:1	12:0	14:0	16:0	16:1	18:0	18:1	18:2	
	Mean±SD									
Control	3.05±0.02	1.29±0.02	4.63±0.36	3.85±0.39	22.52±1.07	9.57±0.31	9.93±0.19	17.49±0.10	27.66±1.14	0.82
With defatted soya flour	3.35±0.37	1.57±0.03	3.35±0.41	3.46±0.05	23.17±0.73	9.86±0.32	9.44±0.14	17.95±1.26	27.85±1.32	0.84
With soya flour	2.75±0.06	1.07±0.01	2.83±0.03	3.04±0.05	21.81±0.70	11.46±.02	7.95±0.43	19.48±0.96	29.62±0.65	0.90

The values are means of three independent experiments

SD Standard deviation

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