

Fermentation by *Trichosporon cutaneum* IFFI01367 for Bio-oil Production from Corn Straw Hydrolysate

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(Received: 11 January 2014; accepted: 26 March 2014)

Medium compositions and fermentation conditions for bio-oil production by fermentation of corn straw hydrolysate with IFFI01367 strain were explored, and the lipid composition was identified. The optimal conditions were determined as follows: 5% sugar, 1% yeast extract as nitrogen source, initial pH 5.8, 30°C, 150 r/min, 50 mL corn straw hydrolysate in 300 mL erlenmeyer flask, fermentation time 4 d. Under these conditions, the biomass reached 23.23g /L, oil content was 42.32% and the oil production capacity was 9.83g /L. The main fatty acids of the oil induced palmitic acid, oleic acid and stearic acid; linoleic acid was more rich, which is convenient to post-processing.

Key words: Corn straw, Hydrolysate, Fermentation conditions, Biological oil.

Energy shortage and environmental pollution bring dual pressures to sustain development in today's society. Some researchers believe that fossil fuels likely replaced by renewable and environmentally friendly fuel in the near future will alleviate this problem (Demirbas 2007; Fjerbaek *et al.* 2009; Jin *et al.* 2013; Liang *et al.* 2013). Biodiesel have a similar nature to fossil diesel fuel, but the contaminated gas emissions from burning fossil diesel was much lower than that from fossil diesel fuel (Kalscheuer *et al.* 2006). Biodiesel generally derived from a variety of animal and plant oils through esterification or transesterification process, most of microbial oil fatty acid are similar to general vegetable oils, which composed of C16 and C18 fatty acid. Therefore, microbial oil can substitute vegetable fats to product biodiesel in

order to ease the global energy crisis (Liu and Zhao 2007; Ferella *et al.* 2010; Cao *et al.* 2012; Liu *et al.* 2012; Fedosov *et al.* 2013).

Many scholars have done a lot of researches on microbial oil. In order to expand the sources of fats fermentation substrate, glycerol (Meesters *et al.* 1996; Saenge *et al.* 2011), lignocellulose (Zhao and Hu 2011), starch wastewater (Xue *et al.* 2010), Jerusalem artichoke (Hua *et al.* 2007), molasses (Alvarez *et al.* 1992), wastewater (Angerbauer *et al.* 2008) and others were used to transform synthetic oils. The sources of lignocellulose have advantages such as a rich source, large numbers of breeds and short regeneration time. Currently, the most effective way to reduce cost is to take advantage of most abundant lignocellulosic resources and industrial wastes in China, such as corn stalks, sorghum bar, straw, wheat straw, bagasse and agricultural processing industry, paper industry generated waste and others (Zhao and Hu 2011). In recent years, with the rapid development of microbial oils, *Trichosporon fermentans* as a oil producing

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microbe strain receives more and more attention, which can use tapioca starch, rice straw hydrolyzate and other substrates to ferment microbial oil (Shen *et al.* 2007; Yuan *et al.* 2011). Chen *et al.* (2009) found that *Trichosporon fermentans* with stronger oil producing ability from the nearly ten oil-producing microbes taking glucose as the sole carbon source, the oil content was more than 30%. In addition, *Trichosporon cutaneum* exhibits strong resistance to some inhibitors such as formic acid, furan compounds and aromatic compounds in addition to the ability to decompose heavy metals, which has great advantages compared with the production of microbial oil taking some cellulose hydrolyzate as substrates.

In the present study, we used *Trichosporon cutaneum* IFFI01367 as fermentation strain, corn stover hydrolyzate as the carbon source, Shake flask fermentation culture condition by *Trichosporon fermentans* were optimized, and the composition and the main physical and chemical properties of the biodiesel from its oil production were analyzed.

MATERIALS AND METHODS

Trichosporon fermentans IFFI01367 strain was purchased from Chinese Institute of Food and Fermentation Industry. Corn stover with cutting crushing for 30-40 mesh was from suburbs of Xinxiang, China, goods xylanase enzyme and cellulase from Shanghai Jienenke Company (Shanghai, China), xylanase activity was 1.76×10^6 U and 2.07×10^6 U for cellulase activity.

Preparation of corn stover hydrolyzate (saccharification liquid)

According to the previous reports (Feng *et al.* 2011), 10g of corn stalk powder was weighed into 300mL flask, diluted with 1% hydrochloric acid to pretreat at 121°C for 60min, added distilled water and adjusted the pH to 5.0, the liquid-solid ratio was 10:1, MgCl_2 was 2.5 nmol/L, Tween 80 was 1.5 mL/L, then added 2.5 g/100 g cellulase and 2.5 g/100 g xylanase, saccharification at 50°C for 48 h, filtered through ordinary filter paper, hydrolyzated for further use.

Preparation of seed solution

Appropriate amounts of bacteria were picked from the activated IFFI01367 slope,

vaccinated 50 mL seed solution on 300 mL flask culture, shaking culture for 22 h, the culture temperature was 28°C, and rotation speed was 150r/min.

Culture for fermentation and fat production

A certain volume of corn stover hydrolyzate was loaded to 300 mL Erlenmeyer flask to sterilize at 121°C for 20 min, then the seeds was transferred to the corn stover hydrolyzate (inoculum was 10%) under cultivation condition of pH5.5, 150r/min and 28°C for some time. Zymotic fluid was centrifugated at 4500 r/min for 10 min, cells were collected and dried (drying temperature was 60°C) to constant weight for use.

Trichosporon yeast oils extraction

According to the previous reports (Hu *et al.* 2011), a amount of crushed dry cells were accurately weighed, 40mL hydrochloric acid was added to per gram of dry cell, placed at room temperature without shaking for 20 min, heated in boiling water for 10 min, the right amount of chloroform-methanol (volume ratio of 2:1) was added for shaking culture for 30 min, then centrifugated at 4500 r/min for 15-20 min, the chloroform layer was removed and dried in vacuo to obtain grease.

Fatty acid composition analysis

Fatty acid composition was detected by Quality Supervision and Inspection Center of Henan Feed Grain and Oil Products, (Zhengzhou, China).

Conventional methods for determination

Determination of the amount of the biomass was expressed as the quality of dry cells per liter of fermentation solution, oil content per dry gram of cells for fat content, and fat mass per liter of fermentation broth for production capacity (Feng *et al.* 2011).

RESULTS

Effects of dissolved oxygen on the fat cell production

In this experiment, the size of dissolved oxygen was expressed as different liquid amount in 300mL flask, the results were shown in Table 1. From Table 1, bacterial biomass and lipid production capacity were the highest when the liquid volume was 50mL, the bacterial biomass and lipid production capacity were significantly decreased

with the increases of liquid volume, indicating that more dissolved oxygen amount was more conducive to the cell growth and the accumulation of grease, 25mL liquid volume was the most conducive to the growth and accumulation of fat cell, the fat content and lipid production capacity was 22.01% and 2.42 g/L. However, the effect of 50mL was not very different from the 25mL liquid volume. So 50mL liquid volume was chosen from the cost of the production.

Impact of inoculum on bacterial fat production

Inoculum size affected the growth and reproduction speed and productivity of bacteria in shake flask medium. 5%, 10%, 15%, 20%, 30% seed were respectively incubated in 50mL medium (pH5.5) at 28°C and 150 r/min for 4d, the weight of the dry cells and total fat content were measured, the results were shown in Table 2.

From Table 2, it can be seen that the fat content reached up to the highest of 25.56% when the inoculum was 15%, the fat producing capacity was also the highest of 4.55g/L while the biomass reached up to the highest of 18.23 g/L at 20%. These may be caused by excessive inoculum

concentration, cell grew too fast, the viscosity of the culture solution increased, resulting in insufficient oxygen, thus affecting the accumulation of fat. If inoculum size was too small, the fermentation speed was too slow, it would result in too long production cycle. 15% inoculum concentration was selected for the best inoculation.

Effects of carbon concentration on the production of fat cell

Total reducing sugars in the corn stover hydrolyzate was relatively little, which was not conducive to the accumulation of fat in the cells, so carbon source needed to be added to improve the oil content of the cells (Kong *et al.* 2007; Cheng *et al.* 2008). In this experiment, sucrose was taken as a supplemented carbon source, the supplemented mass fractions were 3%, 5%, 8% and 10%. 50 mL of corn stover hydrolyzate was filled in a 300 mL flask with inoculum 15%, pH 5.5, shaking speed of 150 r/min at 28°C fermented for 4d. The bacterial biomass, lipid content and lipid production capacity were measured, the results were shown in Table 3. From Table 3, the carbon and nitrogen ratio in the medium increased with

Table 1. Effect of different fermentation liquid volume affected fat fermentation

Liquid volume (mL)	Biomass (g/L)	Fat content (%)	Fat production capability (g/L)
25	14.67	21.34	3.13
50	14.08	22.01	3.10
100	11.89	20.35	2.42

Table 2. Impact of inoculation on cell growth and fat synthesis

Liquid volume (mL)	Biomass (g/L)	Fat content (%)	Fat production capability (g/L)
5	13.67	22.83	3.12
10	17.20	24.01	4.13
15	17.80	25.56	4.55
20	18.23	20.62	3.76
30	12.91	20.45	2.64

Table 3. Impact of additional different concentrations of sucrose on cell growth and lipid synthesis

Sucrose concentration(%)	Biomass (g/L)	Fat content (%)	Fat production capability (g/L)
0	17.8	25.56	4.55
3	17.91	30.77	5.51
5	18.18	34.71	6.31
8	18.73	31.61	5.92
10	17.29	31.46	5.44

the concentration increasing of sucrose, the weight of dry cell, oil production also increased. When the sucrose concentration reached to 5%, fat content and the ability of fat production to achieved maximum values (34.71% and 6.31 g/L respectively).

Effects of nitrogen source on the fat cell production

Species of the nitrogen also affected the accumulation of fat (Li *et al.* 2007). We designed to add a concentration of 1% yeast extract, ammonium sulfate and urea nitrogen as nitrogen resources, 50 mL of corn stover hydrolyzate was added in 300 mL flask, inoculum size of 15%, 5% sucrose, pH 5.5, 150 r/min, at 28°C fermented for 4 d, cells were collected and oil was extracted. The results were shown in Table 4.

Table 4 showed that yeast extract was taken as nitrogen cell biomass, the fat content and fat yield capacity was the maximum, 20.38 g/L, 38.33%, 7.81 g/L respectively. Urea was better than ammonium sulfate as a nitrogen source, but the biomass, lipid content and lipid production

capacity were relatively low in terms of yeast extract, indicating that the utilization efficiency of 01367 to organic nitrogen such as yeast extract and urea was better than that of inorganic nitrogen, so the organic nitrogen was the best source of nitrogen.

Effect of pH on the production of bacterial lipid

pH also had larger influence on microbial oil production, generally the optimum pH value of producing grease was consistent with its optimum co-growth pH value (Wang *et al.* 2010). Different microbes had different optimum pH of producing oils, the seeds in the flask was transferred to liquid fat production medium (pH values were 4.0, 5.0, 5.5, 6.0, 6.5, 7.0 and 8.0), and shaking cultured at 28°C for 4d, the speed was 150r/min. The results were shown in Table 5.

Table 5, the initial pH value of the fermentation medium ranged from 4.0 to 6.0, the amount of the biomass increased with the increasing pH value. While the initial pH value

Table 4. Impact of additional different concentrations of sucrose on cell growth and lipid synthesis

Nitrogen sources(%)	Biomass (g/L)	Fat content (%)	Fat production capability (g/L)
0	18.18	34.71	6.31
Yeast extract	22.20	38.33	8.51
Urea	19.27	36.07	6.95
Ammonium sulfate	19.49	32.97	6.43

Table 5. Influence of pH on cell growth and lipid synthesis

pH	Biomass(g/L)	Fat content(%)	Fat production capability(g/L)
4.0	12.16	35.52	4.32
5.0	20.70	37.78	7.82
5.5	22.20	38.33	8.51
5.8	23.25	41.12	9.56
6.0	24.84	36.95	9.18
6.5	22.68	36.81	8.35
7.0	13.68	30.48	4.17
8.0	12.60	20.24	2.55

Table 6. Influence of temperature on strains growth and lipid synthesis

Temperature (°C)	Biomass (g/L)	Fat content (%)	Fat production capability (g/L)
25	24.11	38.2	9.21
28	23.25	41.12	9.56
30	23.23	42.32	9.83
35	23.08	37.05	8.55

was within the range of 6.0-8.0, the amount of the biomass, oil content and oil production capacity decreased with the increasing pH value. The amount of the oil content and oil production capacity reached its maximum capacity (41.12% and 9.56g/L, respectively) when pH value was 5.8. Experimental results showed that within the scope of the experiment, the initial pH value of the fermentation medium near to 6.0 was in favor of cell growth and fat synthesis, and also helped to improve the utilization of biomass substrates.

Effect of temperature on fat cell production

Temperature can affect the fat content and composition (Ageitos *et al.* 2011). The activated

bacterial seeds were transferred on the culture medium for lipid production at 27°C, 30°C and 35°C for gradient culture, the results were observed and shown in Table 6.

The results showed that bacterial cells grew well at 30°C, the cell biomass and oil content reached the maximum, the utilization efficiency of the substrate achieved optimum. As shown in, the bacterial cells grew well at 30°C, its bacterial biomass, lipid content and lipid production capacity reached up to 23.08 g/L, 42.32%, 9.83g/L, respectively, indicating that the utilization efficiency of bacterial substrate was the best at this temperature.

Table 7. IFFI01367 fat content and fatty acid composition of cells.

Test items	Structure	Measured results
Myristic acid	$\text{CH}_3(\text{CH}_2)_{12}\text{COOH}$	2.0
Palmitic acid	$\text{CH}_3(\text{CH}_2)_{14}\text{COOH}$	24.4
Palmitelaidic acid	$\text{C}_{16}\text{H}_{30}\text{O}_2$	1.9
Heptadecanoic acid	$\text{CH}_3(\text{CH}_2)_{15}\text{COOH}$	0.2
Heptadecenoic acid	$\text{C}_{17}\text{H}_{32}\text{O}_2$	0.2
Stearic	$\text{CH}_3(\text{CH}_2)_{16}\text{COOH}$	14
Oleic	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$	33.2
Linoleic	$\text{CH}_3(\text{CH}_2)_4(\text{CH}=\text{CHCH}_2)_2(\text{CH}_2)_6\text{COOH}$	19.6
Linolenic	$\text{CH}_3(\text{CH}_2)_4(\text{CH}=\text{CHCH}_2)_3(\text{CH}_2)_3\text{COOH}$	1.0
Eicosanoids	$\text{CH}_3(\text{CH}_2)_{18}\text{COOH}$	0.4
Eicosenoic acid	$\text{C}_{20}\text{H}_{38}\text{O}_2$	0.1
Wood tar acids	$\text{C}_{24}\text{H}_{48}\text{O}_2$	2.7
Lauric	$\text{CH}_3(\text{CH}_2)_{10}\text{COOH}$	0.2



Fig. 1. Fat particles of IFFI01367 mycelium before optimization (100×10)



Fig. 2. Fat particles of IFFI01367 mycelium after optimization (100×10)

Fat production of IFFI01367 from the straw hydrolyzate

Cells were picked from the fermentation broth to prepare smear slide, thermally fixed, one drop of Sudan black B dye stain solution was added to stain for 15 min, poured off the dye, washed with xylene, dehydrated to be colorless, then stained with Safranin for 2 min, washed, dried and observed under microscope. Cells and hyphae were red, fat particles in the bacteria body was blue-black (Feng *et al.* 2011). The fat particles of IFFI01367 mycelium before (fermentation conditions: 300 mL flask filled with 50 mL of corn stover hydrolyzate, inoculum of 10%, pH 5.5, 150 r/min, 28°C for 4 d.) and after optimization (fermentation conditions: 300 mL filled with 50 mL flask corn stover hydrolyzate, inoculum of 15%, supplemented with 5% sucrose, 1% yeast extract, pH 5.8, 150 r/min, 30°C for 4 d) were shown in Fig. 1 and Fig. 2.

Fig. 1 and Fig. 2 showed that the number and size of fat particles for the bacterial cells before optimization was significantly higher than that of after optimization.

Composition analysis of bio oil fatty acid in yeast *Trichosporon*

The fatty acid composition was identified by GC-MS analysis after methyl ester of 01367 biological grease, its main fatty acid composition and content were shown in Table 7.

Table 7 showed that the main fatty acid composition of yeast *Trichosporon* 01367 biological oils: oleic acid (C18:1) content was the highest, accounting for 33.2% of total fatty acids. The remainings were as follows: 24.4% of palmitic acid (C16:0), 19.6% of linoleic acid (C18:2), 14% of stearic acid (C18:0) and others.

DISCUSSION

In this paper, corn stover hydrolyzate was taken as the main carbon source, the composition of the culture medium and fermentation conditions were studied to obtain the best conditions: supplemented with 5% sucrose, 1% yeast extract as nitrogen source, pH 5.8, shaking bottle with fermentation liquid volume of 300 mL flask, 50 mL of corn stover hydrolyzate cultured for 4d. Fermentation cultured under optimal conditions at 28°C, 150 r/min with biomass of 23.23 g/L, the fat

content was 42.32%, and lipid production capacity was 9.83 g/L.

According to the study of Harrington (1986), the ideal material as the substitute of diesel should have the following molecular structure: (1) having a longer carbon straight chain, (2) has one or more double bonds, and the double bond was located in terminal or uniformly distributed in the chain of carbon atoms in the carbon chain, (3) containing a quantity of oxygen, preferably ester, ether or alcohol, (4) having as little or no branching as possible in the molecule structure.

However, previous studies showed that the distribution of fatty acid in yeast was a single mode, most yeast had only C16 and C18 fatty acids, saturated fatty acids were palmitic acid and stearic acid substantially, monounsaturated fatty acid was essentially oleic acid, and linoleic acid was less. The tested composition of the fatty acid in fermentation yeast *Trichosporon* 01368 (Feng *et al.* 2011) was as follows: 41.22% of palmitic acid, 36.62% of oleic acid, 15.47% of stearic acid, while linoleic acid was not detected. The tested composition of the fatty acid in budding cryptococcal was 16:0 palmitic acid for 28%, 18:0 stearic acid for 15%, 18:1 oleic acid for 48% (Ageitos *et al.* 2011). The tested composition of the fatty acid in *Sida*'s yeast was C16:0 palmitic acid for 33% and C18:1 stearic acid for 55% (Ageitos *et al.* 2011). The composition of the fatty acid in yeast *Trichosporon* aS 2.571 was detected to be mainly palmitic acid, oleic acid and stearic acid (Hu *et al.* 2011). Hao *et al.* (2013) found that fatty acid composition was mainly C18:1 (oleic acid), C18:0 (stearic acid) and C16:0 (brown dig acid) in *Trichosporon* yeast ACCC20271 fermentation, their percentages of total fatty acids were 52.1%, 14.2% and 17.2%, respectively. The fatty acid composition analysis produced by bio-oil of yeast *Trichosporon* 01367 in this paper showed that unsaturated fatty acids were very rich, the molecular structure was close to the ideal of diesel alternatives.

The conditions of fat production from corn stalk hydrolyzate fermented by *Trichosporon* yeast was primarily explored, grease composition of yeast organisms were analyzed. The yeast would be genetically modified through strain domestication or molecular biology techniques to increase the oil production in the future study, while detoxification technology of corn stover

hydrolyzate will be actively explored to do further research to improve oil production. Mutagenesis or biological engineering strategy can be used in future to further improve the capability of strains tolerating hydrolysis byproducts, thus effectively utilize lignocellulosic hydrolyzate to produce oil.

ACKNOWLEDGEMENTS

This study was supported by the Focus Research of Economic and Social Development in Xinxiang City, China (ID number: ZG11011).

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