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Microbial oil production by *Mortierella isabellina* from sodium hydroxide pretreated rice straw degraded by three-stage enzymatic hydrolysis in the context of on-site cellulase production

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ABSTRACT

Lignocellulosic single cell oil (SCO) is profound and promising in response to the increasing concerns on sustainable energy supply and environmental protection. Different bioprocesses from sodium hydroxide pretreated rice straw (SHPRS) to SCO were compared and the bioprocess c using the three-stage enzymatic hydrolysis was found to be the most efficient. It had the lowest enzyme input 153.1 FPIU cellulase/g SCO and the shortest time 222 h, but produced 42.0 g dry cell biomass and 23.4 g SCO from 342.0 g dry SHPRS. It had the highest lipid content 55.7%, and its productivities and yields were the highest. This study verified that on-site cellulase production by the mixed culture of *Trichoderma reesei* and *Aspergillus niger* and the three-stage enzymatic hydrolysis have the application value in SCO production from lignocelluloses by *Mortierella isabellina*.

1. Introduction

As the largest producer of rice in the world, China produces 0.22 billion metric ton rice straw each year [1,2]. However, it is usually treated as a sort of waste, being burned everywhere at will [3,4]. This causes serious environmental problems, especially air pollution [3,4]. Therefore, why not make good use of it in a more environment-friendly way such as production of biofuels which potentially help the world kick the oil habit [5]?

Microbial lipids, also regarded as single cell oil (SCO), are more renewable and sustainable resource than plant oils and animal fats, though they are the same in chemistry and all of them could be used as feedstock for oleochemical industry [6–8]. Compared to the latter two, SCO production has many advantages including shorter period, higher productivity, lower land requirement, etc. Moreover, it is not affected by environmental factors such as rainfall, sunlight, temperature and so on. More importantly, SCO production is much easier to be automated in large scale than plant oils and animal fats.

SCO production by oleaginous microorganisms from lignocellulosic materials has received more and more attentions because it does not compete with food production and has many merits mentioned above [9–12]. The largest challenge to SCO production from lignocelluloses is the cost, hindering its commercialization. It's the same as the lignocellulosic ethanol production that the biggest three contributors to the cost of SCO production are pretreatment, cellulase production and enzymatic hydrolysis [13,14]. Therefore, efforts should be made to substantially reduce the costs.

Many oleaginous microorganisms can produce SCO efficiently. Among them, *Mortierella isabellina* was reported to be capable of accumulating considerable amount of lipids, ~80% of dry cell biomass [15,16]. Moreover, *M. isabellina* could utilize a variety of substrates, including monomeric sugars, glycerol, lignocellulosic biomass hydrolysate, etc. In addition, *M. isabellina* has excellent tolerance towards the inhibitors resulted from pretreatment [15]. These advantages enable *M. isabellina* to be ideal candidate for SCO production from cheap agricultural residues.

As the most well-known producer of cellulase, *Trichoderma*

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reesei's cellulase mainly contains three components: cellobiohydrolases (CBH: EC 3.2.1.91), exoglucanases which cleave cellobiose molecules from either the reducing or nonreducing ends of a cellulose chain; endoglucanases (EG: EC 3.2.1.4), cleave internal bonds in the cellulose chain; and β-glucosidases (EC 3.2.1.21), which hydrolyze cellobiose into two glucose molecules [13,17,18]. These components could achieve a complete enzymatic hydrolysis of lignocelluloses [3]. However, the improper composition of *T. reesei*'s cellulase hampers its synergism working well [3,13,14,19]. Hence, a mixed culture system composed of *T. reesei* and *Aspergillus niger* was introduced here to produce cellulase with better composition [13,14,20].

High solid loading is a good strategy to reduce the costs of the bioprocesses for biofuels such as bioethanol because it could increase the product concentration and increase the productivity [21,22]. This could increase the economical competitiveness of lignocellulosic SCO production, which is of great importance to its commercialization. However, high solid loading would bring challenge to the enzymatic hydrolysis. For instance, it always elongates enzymatic hydrolysis time and reduces yield. While the multi-stage enzymatic hydrolysis could solve those problems, shortening enzymatic hydrolysis time and raising yield [15,21]. Three-stage enzymatic hydrolysis has been proven efficient and applicable in the SCO production by *M. isabellina* from steam-exploded corn stover [15].

In this work, rice straw was used as the substrate for the bioprocess to produce SCO. Sodium hydroxide pretreatment was adopted to break down the recalcitrance of rice straw, making it easier to be enzymatically hydrolyzed. Then the sodium pretreated rice straw (SHPRS) was used as inducer for cellulase production and the crude cellulase was applied to the enzymatic hydrolysis directly to realize on-site cellulase production. Three-stage enzymatic hydrolysis was employed to increase the efficiency of the enzymatic hydrolysis of SHPRS with high solid loading. Subsequently, the enzymatic hydrolysates of SHPRS were fermented by *M. isabellina* to produce SCO. This work compared the different bioprocesses from SHPRS to SCO and obtained the most efficient one. Though the similar bioprocess was reported in the previous work [15], rice straw instead of corn stover and sodium hydroxide pretreatment instead of steam explosion were used here and this work is important because different substrates and pretreatments have great influences on subsequent bioprocesses [23,24].

2. Materials and methods

2.1. Sodium hydroxide pretreated rice straw

Rice straw was collected in the autumn of the year 2015 from Wuxi City, Jiangsu Province, China. It was air-dried and stored at 25 °C before use. The method adopted to pretreat rice straw was sodium hydroxide pretreatment [18,25]. Before pretreatment, the stored rice straw was ground using a laboratory mill and sieved to collect the particles less than 2 mm in size. The particles were soaked in 2% NaOH aqueous solution with a ratio of 1:8 (g:mL) and kept at 120 °C for 30 min [4,26]. Then the sodium hydroxide pretreated rice straw (SHPRS) was washed using tap water and distilled water to neutral pH. Subsequently, the washed SHPRS was collected and stored at 4 °C until use. The main composition of the washed SHPRS was as follows (dry biomass): glucan 59.7%, xylan 12.4%, lignin 9.3%, others 18.6%.

2.2. Microorganisms and media

T. reesei and *A. niger* were co-cultured to provide cellulase. *M. isabellina* was used to ferment the enzymatic hydrolysates to produce SCO. Their spores were suspended separately in sterile 15–20% (v/v) glycerol and preserved at −80 °C. They were obtained from the strain collection of Biomass Energy Center at Northwest A&F University.

The medium for preparing seeds of *T. reesei* and *A. niger* had a following composition (50 mL): 0.05 g glucose, 0.05 g peptone, 5 mL Mandels nutrients salts solution, 0.05 mL trace elements solution, 0.005 g Tween 80, 2.5 mL 1 M citrate buffer (for pH 4.8). The composition of the fermentation medium for cellulase production was as follows (g/L): SHPRS (dry material) 30, glucose 1, $(NH_4)_2SO_4$ 6, KH_2PO_4 2, $CaCl_2$ 0.3, $MgSO_4$ 0.3, $FeSO_4$ 0.005, $MnSO_4$ 0.0016, $ZnSO_4$ 0.0014, and $CoCl_2$ 0.0037. The initial pH was adjusted to 4.8 using citrate buffer. All the components except SHPRS were purchased from Sinopharm Chemical Reagent Co. Ltd., Shanghai, China. The media were autoclaved at 121 °C for 20 or 30 min (20 min for the medium without SHPRS and 30 min for the medium with SHPRS) [4,13,14].

The fermentation medium for SCO production had as following composition: the enzymatic hydrolysate of SHPRS, 0.5 g/L $(NH_4)_2SO_4$, 0.5 g/L yeast extract, 0.5 g/L, 7 g/L KH_2PO_4, 2 g/L Na_2HPO_4, 1.5 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L $CaCl_2{\cdot}2H_2O$, 0.008 g/L $FeCl_3{\cdot}6H_2O$, 0.001 g/L $ZnSO_4{\cdot}7H_2O$, 0.0001 g/L $CuSO_4{\cdot}5H_2O$, 0.0001 g/L $Co(NO_3)_2{\cdot}H_2O$, 0.0001 g/L $MnSO_4{\cdot}5H_2O$, the pH was adjusted to 5.5. All the components except the enzymatic hydrolysate of SHPRS were purchased from Sinopharm Chemical Reagent Co. Ltd., Shanghai, China. The medium was autoclaved at 121 °C for 30 min [4,15,26].

2.3. Mixed cultures of T. reesei and A. niger and monocultures

T. reesei and *A. niger* were pre-cultured in the seed medium for 36 h and 48 h respectively to accumulate the satisfactory amounts of biomass before being inoculated into the fermentation medium. For the mixed culture, 10% (v/v) pre-cultured *T. reesei* and 10 or 2% (v/v) *A. niger* were inoculated into each flask. The delay time of *A. niger* inoculation was 0 h (inoculated simultaneously), 24, or 48 h. The different inoculum ratios and the different delay times of *A. niger* inoculation derived 6 mixed culture forms (denoted as 0 h/1:1, 0 h/5:1, 24 h/1:1, 24 h/5:1, 48 h/1:1, and 48 h/5:1) [13,14]. For the monocultures, 10% (v/v) pre-cultured *T. reesei* or10% (v/v) *A. niger* were inoculated into each flask. Then the flasks were incubated with a shaking of 170 rpm at 30 °C on first day and 28 °C since then.

2.4. Enzymatic hydrolysis of sodium hydroxide pretreated rice straw

2.4.1. Comparison of differently sourced cellulases

The comparison of differently sourced cellulases in the enzymatic hydrolysis of SHPRS was conducted in 250 mL Erlenmeyer flasks. The total liquid was 50 mL containing 2.5 mL 1 M citrate buffer (pH 4.8), 100 g/L SHPRS (dry material) and 25 FPIU/g glucan the cellulase, and supplementary amount of distilled water. The cellulases compared in this work included the cellulase produced by the mixed culture of *T. reesei* and *A. niger* (48 h/5:1), the cellulase from the monoculture of *T. reesei*, and Celluclast the commercial cellulase purchased from Sigma Chemicals. After the enzymes were added, the reaction mixtures were incubated in an orbital shaker (140 rpm) at 50 °C for 48 h. The enzymatic hydrolysis processes were monitored by periodic sampling [4,15,27].

2.4.2. One-stage enzymatic hydrolysis of sodium hydroxide pretreated rice straw

The one-stage enzymatic hydrolysis of SHPRS was conducted in 250 mL Erlenmeyer flasks with a working volume 50 mL containing