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Co-utilization of glucose, xylose and cellobiose by the oleaginous yeast *Cryptococcus curvatus*

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ABSTRACT

Simultaneous utilization of mixed sugars is one of the major challenges for biofuel production utilizing lignocellulosic biomass as feedstock. Our previous work proved that the oleaginous yeast *Cryptococcus curvatus* could efficiently produce lipids, the precursors of hydrocarbons with high energy density, from lignocellulosic hydrolysates. However, the strain's capability of simultaneously utilizing primary sugars was still unknown. Thus, this work comprehensively explored the co-utilization of glucose, xylose and cellobiose by *C. curvatus*. The results indicated that the consumption of both xylose and cellobiose was repressed by glucose, while xylose and cellobiose could be simultaneously consumed at similar rates. The total sugar consumption rates remained constant at about 0.6 g L^{-1} h^{-1} regardless of the sugar composition in the mixtures, and the cell biomass and lipid production by *C. curvatus* cultured on the different sugar mixtures were similar. Moreover, compared with glucose and xylose, cellobiose could lead to higher dry cell weights and lipid yields, suggesting an excellent carbon source for lipid production. Based on these findings, this study demonstrated an effective approach for alleviating glucose repression for microbial lipid production by *C. curvatus* through xylose/cellobiose co-utilization which would greatly contribute to a more efficient and economical cellulosic biofuel production.

1. Introduction

Biofuel production using lignocellulosic materials as feedstock has nowadays been of great importance with the increasing concerns of economic feasibility and environmental sustainability [1]. Despite the variations in carbohydrate composition of different lignocellulosic feedstocks, after pretreatment and enzymatic hydrolysis, hexoses and pentoses, such as glucose and xylose respectively, are always the major sugars in lignocellulosic biomass hydrolysates [1]. As a consequence, in the bioconversion process of lignocellulosic biomass, beyond depolymerizing sugars from recalcitrant biomass, another major challenge is an efficient fermentation of sugar mixtures by microorganisms [1]. In the presence of both glucose and xylose, most microorganisms generally metabolize sugars sequentially (first glucose and then xylose) since glucose represses other sugar utilizations mediated by a carbon catabolite repression (CCR) mechanism and/or allosteric competition for sugar transporters [2]. This sequential utilization of the sugars by the microorganisms results in extension of the cultivation period and inefficient utilization of the substrates, thereby decreasing the total productivity [3]. Therefore, the bio-economy of converting lignocellulosic biomass into biofuels can be enhanced by co-utilization of the sugars (e.g. glucose, xylose, etc.) [2].

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To develop the strains capable of simultaneously utilizing both C5 and C6 sugars and to overcome the obstacles resulting from glucose repression, various approaches have been attempted such as (1) modifying the genes involved in the glucose-signalling and regulatory pathway, (2) expressing the genes for the secondary sugar transporters and catabolic enzymes, and (3) controlling culture conditions in a chemostat, such as glucose concentration and dilution rate [1,4]. Among these techniques the bioprocess engineering strategy, called "glucose-limited fed-batch", has been carried out, but it was found to be impractical at an industrial scale when lignocellulosic biomass was utilized as feedstock because glucose is the most abundant monosaccharide in lignocellulosic biomass [1]. Recently, another new metabolic engineering method has been established by introducing a cellodextrin transporter and an intracellular β-glucosidase (BGL) into host microorganisms with the advantage of little glucose accumulation in the medium, resulting in an effective co-fermentation of cellodextrin and non-glucose sugars [1]. By applying this strategy, Ha et al. [5] & [6] successfully constructed the engineered *Saccharomyces cerevisiae* which could co-ferment cellobiose and xylose as well as cellobiose and galactose simultaneously and the strain exhibited improved ethanol yields.

During the past few years, co-fermentation of mixed sugars has been intensively investigated for ethanol production, but studies on oleaginous microorganisms for microbial lipid production have been quite limited. Oleaginous microorganisms have the capability to accumulate intracellular lipids to at least 20 wt.% of dry cell weight, and their fatty acid compositions are similar with those found in plant oils [7]. Thus oleaginous yeasts are suggested as good candidates for lipid-based biofuel production [8]. Actually, the capability of simultaneous sugar utilization greatly varies with different strains and also depends on culture conditions. Gong et al. [9] demonstrated that *Lipomyces starkeyi* simultaneously utilized cellobiose and xylose at their mass ratios of 2:1 and 1:1, while Hu et al. [2] found that *Trichosporon cutaneum* could consume glucose and xylose in a simultaneous pattern at their mass ratios of 1:2 and 1:1. Among the well-studied oleaginous yeasts, *Cryptococcus curvatus* has also emerged as an ideal lipid-producing cell factory because its lipid content can reach up to 83 wt.% of dry cell weight with great performance for the utilization of a variety of substrates [10,11]. Our previous work revealed that *C. curvatus* was able to accumulate microbial lipids by using the hydrolysate from dilute acid pretreatment of wheat straw [12]. However, the ability of *C. curvatus* to effectively utilize mixed sugars has not been studied yet.

Therefore, the aim of this work was to evaluate co-utilization of glucose, xylose and cellobiose by *C. curvatus* for lipid production in order to design a more efficient cultivation using lignocellulosic hydrolysates. Firstly, the performance of *C. curvatus* was examined on the mixtures of glucose/xylose, glucose/cellobiose and cellobiose/xylose in terms of sugar consumption as well as cell biomass and lipid production. Then, *C. curvatus* was cultivated in a sugar mixture made up of cellobiose, xylose and small quantities of glucose to further investigate mixed sugar utilization. This study comprehensively characterized the utilization patterns of glucose, xylose and cellobiose of *C. curvatus*, and provided an effective strategy to minimize glucose repression on xylose consumption through the co-utilization of cellobiose and xylose for microbial lipid production.

2. Materials and methods

2.1. Media and yeast strain preparation

C. curvatus (ATCC 20509) was kept at −80 °C and recovered in YPD medium sterilized by autoclaving at 121 °C for 20 min containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose at 30 °C in an orbital shaker at 150 rpm for 24 h as a pre-culturing step.

2.2. Lipid production on the mixtures containing two sugars

The culture media were prepared as follows: 70 g L^{-1} carbon source (glucose, D-xylose, cellobiose, or a mixture of any two sugars), 0.4 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2.0 g L^{-1} KH_2PO_4, 0.003 g L^{-1} $MnSO_4 \cdot H_2O$, 0.0001 g L^{-1} $CuSO_4 \cdot 5H_2O$, and 5.6 g L^{-1} yeast extract.

2.3. Lipid production on the mixture containing glucose, xylose and cellobiose

The culture media were prepared as follows: 40 g L^{-1} cellobiose, 20 g L^{-1} D-xylose, 10 g L^{-1} glucose, 0.4 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2.0 g L^{-1} KH_2PO_4, 0.003 g L^{-1} $MnSO_4 \cdot H_2O$, 0.0001 g L^{-1} $CuSO_4 \cdot 5H_2O$, and 5.6 g L^{-1} yeast extract.

In all experiments, the culture media with pH adjustment to 5.5 were sterilized by filtration through a 0.45 μm membrane (Millipore Corp., MA). Seed inoculums (10%, v/v) from the pre-culturing step were introduced to the culture media after washing with an equal volume of sterile distilled water twice resulting in a starting cell biomass of about 0.8 g L^{-1} and cultures were maintained at 30 °C in an orbital shaker at 180 rpm with a working volume of 50 mL in 250 mL Erlenmeyer flasks unless mentioned otherwise. All experiments were conducted in triplicate.

2.4. Analytical methods

2.4.1. Dry cell weight determination and fatty acids analysis

A 5 mL cell suspension sample was taken and centrifuged at 2500 rpm for 5 min followed by being washed twice with distilled water. Finally the samples were dried in a pre-weighed aluminium dish at 105 °C to constant weight. The cell biomass was expressed as dry cell weight (DCW, g L^{-1}).

For fatty acid analysis, the yeast cells were harvested, washed twice with distilled water and freeze-dried overnight. Afterwards the fatty acids in the samples were converted to fatty acid methyl esters (FAMEs) by a two-step reaction according to O'Fallon et al. [13]. Qualitative and quantitative analyses of FAMEs were then performed as described by Dong et al. [14] on an Agilent 7890A gas chromatography equipped with an auto-sampler (Agilent 7683B), a flame ionization detector (FID) and a FAMEWAX column (30 m × 320 μm × 0.25 μm) (Restek Corp., PA). Helium was used as the carrier gas. The injector was kept at 280 °C with an injection volume of 1 μL with