



Deficiency of β -Glucosidase Beneficial for the Simultaneous Saccharification and Lipid Production by the Oleaginous Yeast *Lipomyces starkeyi*

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Abstract

It is inevitably for cellobiose to be co-generated during enzymatic hydrolysis of cellulose, especially when the cellulase is lack of β -glucosidase activity. In the present study, cellobiose was found superior to glucose for cell growth by *L. starkeyi*, regardless of the sugar concentrations. Glucose was assimilated preferentially when cellobiose and glucose were co-fermented. Deficiency of β -glucosidase was observed to be beneficial for the simultaneous saccharification and lipid production (SSLP). High lipid titer and cellulose conversion of 9.1 g/L and 92.4%, respectively, were achieved when cellulase with low β -glucosidase activity was supplemented. The SSLP achieved higher lipid titer of 9.5 g/L when a pre-hydrolysis process was introduced. The glucosidase generated by *L. starkeyi* was primarily cell-bound, which contributed significantly to the cellobiose utilization and the high lipid production. These results provided a novel scheme for enhanced lipid production from lignocellulosic biomass with reduced enzyme usage, which is believed to facilitate the design of a more cost-effective lignocellulose-to-lipid route.

Keywords Lignocelluloses · Cellobiose · *Lipomyces starkeyi* · Simultaneous saccharification and lipid production · Microbial lipid

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Introduction

Lignocelluloses-derived microbial lipid prepared from the oleaginous species has emerged as a promising candidate to address the shortage of oil source for biodiesel industry [1, 2]. Lignocelluloses are naturally comprised of cellulose, hemicelluloses, lignin, among others [3]. Lignocellulosic sugars include glucose, xylose, arabinose, galactose, mannose, cello-oligosaccharides, and xylo-oligosaccharides [4]. Some oleaginous yeasts, with wide substrate spectrum, can assimilate these sugars for lipid production [5, 6].

In general, lipid production from lignocelluloses involves three major steps, namely pretreatment to overcome the biomass recalcitrance, enzymatic hydrolysis to release fermentable sugars, and fermentation to overproduce microbial lipids [7]. Hydrolysis process requires various enzymatic components to realize synergistic effect [8]. Specifically, for efficient hydrolysis of cellulose, synergistic action of three types of enzymes including exoglucanase, endoglucanase, and β -glucosidase are prerequisite for complete conversion of cellulose into glucose [9, 10]. It is worth noting that the intensively applied *Trichoderma reesei* secretes cellulase enzyme complex with very low proportion of β -glucosidase [10, 11]. When cellulose is hydrolyzed by this type of cellulase, cellobiose and even higher degree of oligosaccharides are routinely co-generated. Therefore, complete hydrolysis of cellulose into glucose generally necessitates substantial supplementation of *T. reesei* cellulase with a commercial β -glucosidase [12]. High enzyme cost remains a challenge that causes hindrance to the techno-economic of the lignocellulose-to-lipid bioprocesses.

Cellobiose, the basic structural unit of cellulose, has been taken as an appropriate carbon and energy source for lipogenesis by some oleaginous species including *Lipomyces starkeyi* and *Cryptococcus curvatus* [5, 13]. Oleaginous yeasts that are capable of effective utilization of cellobiose to produce microbial lipid possess two advantages. On the one hand, oleaginous yeast featuring oligosaccharide assimilation capacity facilitates full utilization of sugars released from lignocelluloses for lipogenesis. On the other hand, the simultaneous saccharification and lipid production (SSLP) process may be efficiently performed without β -glucosidase supplementation. Glucose and cellobiose represent the two major inhibitory products of cellulase, which dramatically lower the saccharification rate. The feedback inhibition by glucose can be avoided while the cellobiose inhibition remains, when the simultaneous saccharification and fermentation (SSF) is performed by the microorganisms without cellobiose-metabolism capacity [14]. The supplementation of extra β -glucosidase is proposed to prevent cellobiose inhibition [14–16].

It is generally known that the SSF integrating enzymatic hydrolysis and fermentation processes can reduce investment cost, avoid end product inhibition of the enzymatic hydrolysis, and require less enzymes [7, 17]. The enzyme cocktail supplementation can be further simplified during the SSF when the microorganisms can be effective in assimilating cellobiose [7, 18–20]. In the present study, cellobiose was observed to be more conducive than glucose to cell growth of *L. starkeyi*. The SSLP process was then conducted and compared by *L. starkeyi* using cellulase with low or high β -glucosidase activity. Deficiency of β -glucosidase was identified as conducive to the SSLP with improved lipid production and cellulose conversion. This SSLP can reduce the investment cost, lower the enzymes cost, minimize the loss of oligosaccharides, and enhance the lipid production, which provides valuable information for the design of a more cost-effective lignocellulose-to-lipid route.

Materials and Methods

Strain and Media

Sigmacell cellulose type 101 was purchased from Sigma-Aldrich and dried in an oven at 105 °C to a constant weight prior to use. Cellulase derived from *T. reesei* (*T*-cellulase) was purchased from Sigma-Aldrich. The filter paper activity and cellobiase activity were determined to be 113.6 FPU/mL and 5.7 CBU/mL, respectively (Table 1). Cellic® CTec2 was supplied by Novozymes (Tianjin, China). The filter paper activity and cellobiase activity were 260.6 FPU/mL and 4167.0 CBU/mL, respectively (Table 1). The *p*-nitrophenyl- β -D-glucopyranoside (pNPG) was purchased from Sigma-Aldrich. Other analytical grade reagents were purchased locally.

L. starkeyi AS 2.1560 used in this study was sourced from the China General Microbiological Culture Collection Center (CGMCC). It was propagated twice a month on yeast peptone dextrose (YPD) slope consisting of 10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose, and 18 g/L agar. Pre-cultures were prepared from 50 mL YP liquid medium (10 g/L yeast extract, 10 g/L peptone) containing 19.0 g/L cellobiose or 20 g/L glucose at 30 °C for 40 h to achieve the late-exponential growth phase unless otherwise stated.

Lipid Fermentation on Glucose or Cellobiose

Lipid fermentation was performed in 250-mL Erlenmeyer flasks containing 5 mL of pre-cultures and 45 mL of the specified medium. The cultures were maintained at 30 °C and a shaking frequency of 200 rpm in an orbital shaking incubator unless otherwise stated. The pH was adjusted to 6.0 at 24-h intervals. The lipid fermentation medium contained 1.0 g/L (NH₄)₂SO₄, 1.0 g/L yeast extract, 1.0 g/L KH₂PO₄, and 1.0 g/L MgSO₄·7H₂O and the addition of glucose, cellobiose, or the mixed sugars at varying concentrations.

SSLP Procedure

SSLP was conducted at 30 °C and 200 rpm in 250-mL Erlenmeyer flasks containing 50 mL medium. Ten percent (w/v) of pre-cultures was inoculated, and the SSLP was held for 144 h. *T*-cellulase or Cellic® CTec2 of 15 FPU/g was loaded at the beginning of the culture. The SSLP medium contained 50 g/L cellulose, 1.0 g/L (NH₄)₂SO₄, 1.0 g/L yeast extract, 1.0 g/L KH₂PO₄, and 1.0 g/L MgSO₄·7H₂O. The initial pH was set to 5.0 and adjusted to the original value at 24-h intervals. For the pre-hydrolysis process, the cellulose medium was sterilized; supplemented with 15 FPU/g *T*-cellulase; and hydrolyzed at 50 °C, pH 4.8, and 200 rpm for 12 h. The medium was then cooled to 30 °C; inoculated with 10% (w/v) of pre-cultures; and held at 30 °C, pH 5.0, and 200 rpm for 144 h. All media were subjected to sterilization by autoclaving at 121 °C for 20 min prior to use.

Table 1 Filter paper activity and cellobiase activity of two types of cellulase

Entry	Filter paper activity (FPU/mL)	Cellobiase activity (CBU/mL)	CBU/FPU
<i>T</i> -cellulase	113.6	5.7	0.05
Cellic® CTec2	260.6	4167.0	15.99

Enzymatic Hydrolysis of Cellulose

The enzymatic hydrolysis of cellulose medium containing 50 g/L cellulose, 1.0 g/L $(\text{NH}_4)_2\text{SO}_4$, 1.0 g/L yeast extract, 1.0 g/L KH_2PO_4 , and 1.0 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was performed at 30 °C and 200-rpm shaking. The initial pH was set to 5.0 and adjusted to the original value at 24-h intervals. *T*-cellulase or Cellic® CTec2 was separately supplemented at 15 FPU/g cellulose. The enzymatic hydrolysis was held for 144 h. In addition, 3 g/L sodium azide was added to prevent bacterial contamination.

Analytical Method

A biosensor analyzer (SBA-40E, Jinan, China) was employed for the determination of glucose. The dinitrosalicylate (DNS) method was applied for the total reducing sugars (TRS) measurement according to a published procedure [21].

Cellobiose and cello-oligosaccharides were determined in accordance with a published method with some alterations [22]. Firstly, the sample was diluted with water to a typical sugar concentration varying from 0.2 to 10 g/L. Two milliliters of the appropriately diluted sample and 69.7 μL of 72% (w/w) H_2SO_4 were added into a 10-mL screw-cap glass bottle to 4% (w/w) H_2SO_4 . The glass bottle was then sealed, placed in an autoclave, and maintained at 121 °C for 1 h. The identical treatment was performed for 1-g/L glucose recovery standard. The sample was then neutralized with CaCO_3 powder to pH between 5 and 6 when the reaction was complete. The liquid sample was collected by centrifugation. The glucose concentrations of the samples before and after the reaction were measured. The cellobiose and cello-oligosaccharides were calculated using the following equation:

$$c_{[\text{cellobiose}]} (\text{g/L}) = \frac{[c'_{[\text{glucose}]} (\text{g/L}) \times v'_{[\text{glucose}]} (\text{L}) / R_{[\text{glucose}]} - c_{[\text{glucose}]} (\text{g/L}) \times v_{[\text{glucose}]} (\text{L})]}{v_{[\text{glucose}]} (\text{L})} \times r$$

$c'_{[\text{glucose}]}$ and $v'_{[\text{glucose}]}$ represent the concentration and volume of glucose after hydrolysis, while $c_{[\text{glucose}]}$ and $v_{[\text{glucose}]}$ denote those before hydrolysis. $R_{[\text{glucose}]}$ indicates the recovery of glucose during the hydrolysis at 121 °C using 4% (w/w) H_2SO_4 . The coefficient r is 0.95. The coefficient r is 0.90 for cello-oligosaccharides.

Cellulose was measured by the phenol sulfuric acid method with some modifications [23]. Specifically, 10 mL of the culture broth was centrifuged (8000 rpm, 5 min) to remove the supernatant. The sediment was resuspended in 3 mL of acetic acid-nitric acid mixture, mixed thoroughly, and processed in a boiling water bath for a period of 30 min. The sample was then centrifuged to get rid of the supernatant. The precipitate was washed twice with water before drying in an oven at 105 °C overnight. The sample was mashed with a glass rod before 10 mL of 67% (w/v) sulfuric acid was added. The reaction occurred at 30 °C for 1 h. The resulting solution was diluted appropriately, and the total sugar was measured using the phenol-sulfuric acid method [24].

The activity of aryl- β -glucosidase was determined as follows. Briefly, wet cells were harvested by centrifugation (8000 rpm, 5 min, 4 °C), and the supernatant was involved to analyze the aryl- β -glucosidase activity in accordance with a published method [5]. The wet cells were washed four times with phosphate-citrate buffer (pH 6.0) and resuspended in the same buffer. The cell suspension was subjected to the determination of cell bound hydrolytic activity.

The total mass containing lipid-rich cells and residual cellulose was collected by centrifugation (8000 rpm, 5 min), washed twice with water, and determined gravimetrically after drying in an oven to the constant weight. Cell mass, expressed as cell dry weight (CDW), was determined by deducting the residual cellulose from the total mass.

The total lipid was extracted using a published chloroform-methanol method [13]. Lipid titer was determined by gravimetric method. Lipid yield was calculated as gram lipid produced per gram cellulose consumed.

All experiments were conducted in triplicate, and the data presented were the average of three independent experiments.

Results and Discussion

Cell Growth on Cellobiose and Glucose

Cellobiose is inevitable to be co-generated during enzymatic hydrolysis when the enzyme blends are lacking in β -glucosidase activity, as the β -glucosidase component is responsible for hydrolyzing soluble cellodextrins and cellobiose to glucose [12, 16]. Large amounts of oligosaccharides were released when the high solid loadings were imposed during enzymatic hydrolysis [4]. Cellobiose is more challenging sugar than glucose for microorganisms, because utilization of cellobiose necessitates additional β -glucosidase and sugar transporters [25]. Oleaginous species capable to assimilate cellobiose for lipid fermentation are conducive to the techno-economics of the lignocellulose-to-lipid bioprocess.

Here, cell growth on cellobiose or glucose by *L. starkeyi* under identical carbon molar concentrations and culture conditions was investigated. As depicted in Fig. 1, the cell densities were consistently higher on cellobiose than on glucose during the culture and finally reached 10.0%, 10.9%, and 16.0%, respectively, higher than those of the latter. It was indicated that cellobiose was preferable to glucose for cell growth, regardless of the sugar concentrations. These data were consistent with that cellobiose was assimilated faster than glucose when *L. starkeyi* was cultured in 70 g/L of cellobiose or glucose [13]. *L. starkeyi* with powerful cellobiose metabolism is valuable for full conversion of lignocellulosic sugars to lipids. Some oleaginous species have possessed the capability of cellobiose consumption, which was essential for lignocellulosic biomass biotransformation [5, 6]. In addition, ethanol production on cellobiose has been realized by engineered *Saccharomyces cerevisiae* expression of a high-affinity cellodextrin transporter and an intracellular β -glucosidase [25–27].

As glucose and cellobiose generally co-existed in the biomass hydrolysates, co-fermentation of glucose and cellobiose by *L. starkeyi* was investigated. The sugars consumption profiles illustrated in Fig. 2 clearly showed that glucose was assimilated rapidly, while cellobiose was consumed very slowly, regardless of the pre-cultures prepared from the seed medium with glucose (Fig. 2a) or cellobiose (Fig. 2b) as the sole carbon source. It was demonstrated that *L. starkeyi* assumed a sequential substrate consumption manner with a strong preference over glucose. Similar phenomenon was observed when glucose and xylose co-existed, suggesting the occurrence of glucose repression [13]. Interestingly, cellobiose was almost consumed up at the end of the culture, suggesting the cellobiose metabolism was robust.

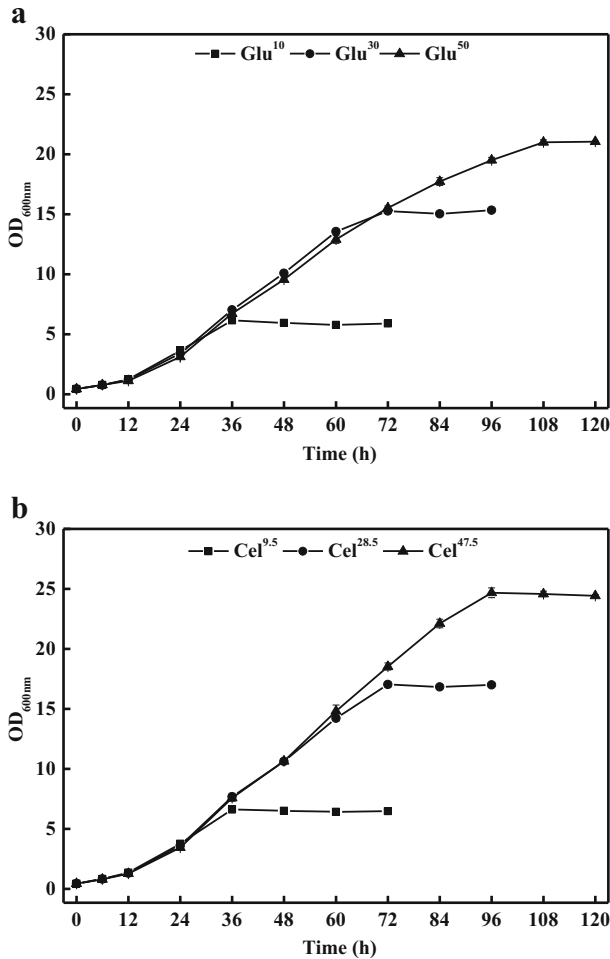


Fig. 1 Comparison of cell growth on various concentrations of glucose (**a**) or cellobiose (**b**) as sole carbon source by *L. starkeyi*. Glucose and cellobiose are abbreviated as Glu and Cel, respectively. Initial sugar concentrations (g/L) are indicated by superscripts after the sugar abbreviations. Glucose and cellobiose were provided to ensure the comparison trials initially containing equimolar concentrations of carbon. The culture was conducted at 30 °C, pH 6.0, and 200 rpm. Error bars represent standard deviation from the mean of three independent experiments

SSLP on Cellulose Without Supplementing β -Glucosidase

The SSLP on 50 g/L cellulose by *L. starkeyi* using Cellic® CTec2 or *T*-cellulase was investigated and compared. The profiles of substrate consumption, cell growth, and lipid accumulation are illustrated in Fig. 3. Cellulose was constantly decreased while the lipid accumulated concurrently. Glucose was increased to 3.2 g/L at 24 h when Cellic® CTec2 was loaded. Glucose and cello-oligosaccharides were kept at very low concentrations, when the SSLP culture was in excess of 48 h. Lipid titer and cellulose conversion were 7.7 g/L and 89.6%, respectively, when the SSLP was stopped at 144 h (Fig. 3a). In contrast, cello-oligosaccharides reached 3.9 g/L at 24 h when the *T*-cellulase with low β -glucosidase activity

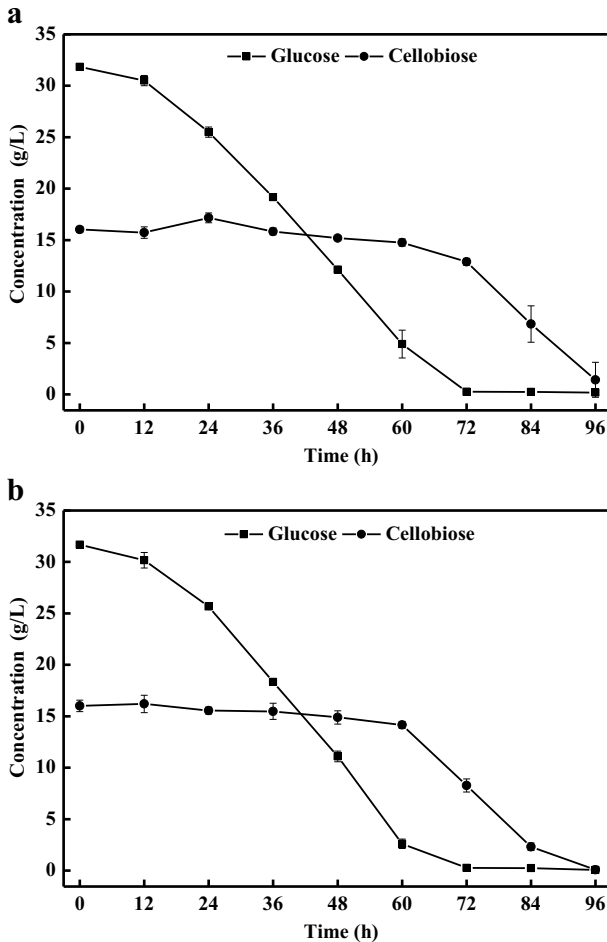


Fig. 2 Sugar mixture consumption profiles by *L. starkeyi* on a mixture of 33.3 g/L glucose and 15.8 g/L cellobiose. Pre-cultures were prepared from YP liquid medium containing 20 g/L glucose (a) and 19.5 g/L cellobiose (b), respectively. The culture was conducted at 30 °C, 200 rpm for 96 h. Error bars represent standard deviation from the mean of three independent experiments

was used. Cello-oligosaccharides were then reduced and consistently below 0.5 g/L when the SSLP exceeded 24 h. Surprisingly, lipid titer and cellulose conversion reached 9.1 g/L and 92.4%, respectively (Fig. 3b). It was indicated that high amounts of β -glucosidase exerted no positive effects on the overall cellulose conversion and lipid output.

Enzymatic hydrolysis of cellulose with these two types of cellulases under the identical conditions of the SSLP cultures was investigated. Cello-oligosaccharides were observed in small amounts when the enzymatic hydrolysis of cellulose was performed with Cellic® CTec2 enzyme blend with high β -glucosidase activity. Glucose and cello-oligosaccharides were 25.5 g/L and 1.5 g/L, respectively, corresponding to 45.9% and 3.0% of the theoretical values, when hydrolysis was terminated at 144 h (Fig. 4a). In sharp contrast, cello-oligosaccharides were constantly accumulated during the hydrolytic process when the hydrolysis was loaded with *T*-cellulase (Fig. 4b). Glucose and cello-oligosaccharides were 10.9 g/L and 7.0 g/L, respectively,

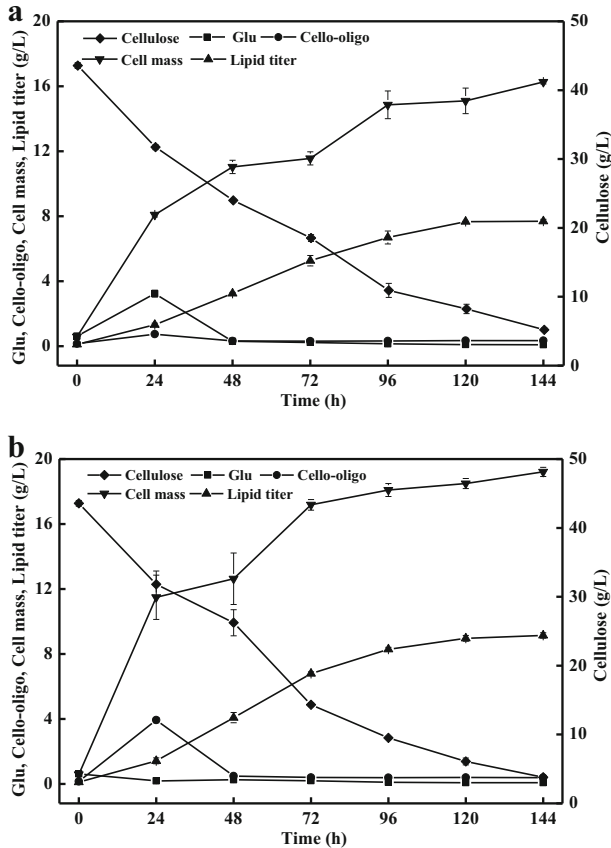


Fig. 3 SSLP of 50 g/L cellulose by *L. starkeyi* using Cellic® CTec2 (a) and *T*-cellulase (b) loaded at 15 FPU/g cellulose. The culture was held at 30 °C, pH 5.0, and 200 rpm for 144 h. Glucose and cello-oligosaccharides are abbreviated as Glu and Cello-oligo, respectively. Error bars represent standard deviation from the mean of three independent experiments

corresponding to 19.7% and 14.0% of the maximum theoretical values (Fig. 4b). The deficiency of β -glucosidase activity was responsible for the accumulation of cello-oligosaccharides. Obviously, the product inhibition was more severe when oligosaccharides were accumulated. Cellulose conversion rates for Cellic® CTec2 and *T*-cellulase at the optimal temperature and pH were 77.3% and 61.1%, respectively (data not shown). It was worth mentioning that significantly higher proportion of cellulose was hydrolyzed by Cellic® CTec2 or *T*-cellulase when the hydrolysis and lipid fermentation were integrated, indicating that the hydrolysis could be significantly accelerated. Surprisingly, higher cellulose conversion was observed using *T*-cellulase than using Cellic® CTec2 during the SSLP, which suggested that the released oligosaccharides were instantly assimilated by *L. starkeyi*.

Aryl- β -glucosidase activity was measured to investigate the cellobiose hydrolytic activities during the SSLP process loaded with *T*-cellulase. As illustrated in Fig. 5, the aryl- β -glucosidase activity of the cell-free broth was low and slowly increased over time. The maximal activity was merely 19.4 U/L at 144 h. Surprisingly, for cell suspension, the aryl- β -glucosidase activity increased rapidly and reached 475.8 U/L at 120 h. This result

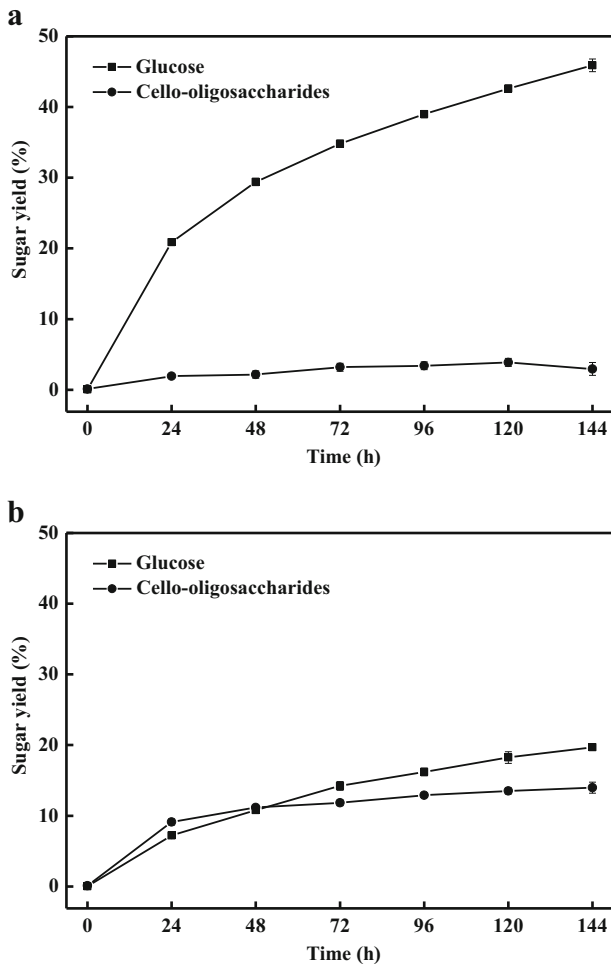


Fig. 4 Sugar evolution profiles during the enzymatic hydrolysis of cellulose using Cellic® CTec2 (**a**) or T-cellulase (**b**) loaded at 15 FPU/g cellulose. The enzymatic hydrolysis was held at 30 °C, pH 5.0, and 200 rpm for 144 h. Error bars represent standard deviation from the mean of three independent experiments

was 23.5 times higher than that of the cell-free broth, indicating that the glucosidase generated was primarily cell-bound with only a minor amount secreting to the environment. It was suspected to be the major reason why SSLP could be effectively conducted by *L. starkeyi* without external β -glucosidase. Inducible expression of glucosidases occurred when cellobiose was used as the sole carbon sources (data not shown). The production of glucosidase meant the additional consumption of nitrogen source within the medium, which created an external environment with a rising carbon-to-nitrogen (C/N) ratio. Lipid accumulation of oleaginous yeasts was stimulated by nitrogen starvation as a routine [28]. Lipid biosynthesis was significantly affected by the C/N ratios of the culture media for *L. starkeyi* [29]. Thus, the additional glucosidase expression was possible to contribute significantly to the higher lipid production.

The major bottlenecks during enzymatic hydrolysis lie in glucose and cellobiose inhibition, which exert negative effects on the hydrolysis efficiency and yield. Recently, the SSLP has

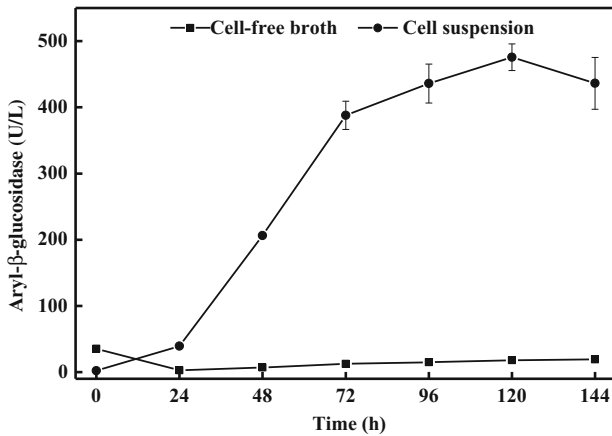


Fig. 5 Time course of aryl- β -glucosidase activity during SSLP of *L. starkeyi* using *T*-cellulose. Error bars represent standard deviation from the mean of three independent experiments

been developed to circumvent glucose inhibition and reduce enzyme usage [7, 30]. Superior lipid production has been made possible using the SSLP as compared with the separate hydrolysis and fermentation process [30, 31]. Here, both glucose and cellobiose inhibitions were avoided during the SSLP using the *T*-cellulase with low β -glucosidase activity by *L. starkeyi*, which was pivotal for better techno-economics of the cellulosic lipid industry. Similar results have been observed by *C. curvatus* using the SSLP without β -glucosidase supplementation [7].

Pre-Hydrolysis and SSLP for Lipid Production

Pre-hydrolysis step has been routinely involved prior to the simultaneous saccharification and fermentation to foster superior production of bioproducts [32–34]. The sugars were very low

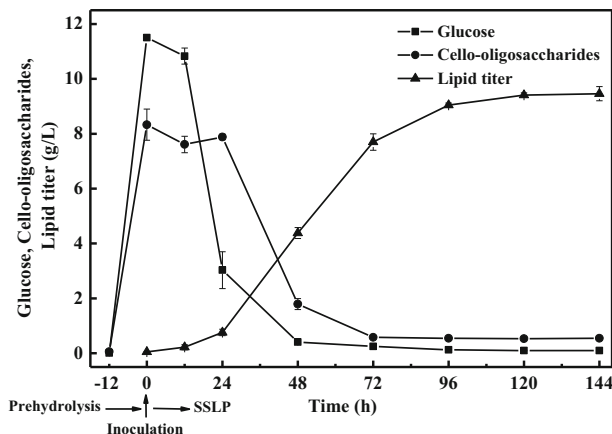


Fig. 6 Sugars and lipid evolution profiles of SSLP by *L. starkeyi* with a pre-hydrolysis step. The pre-hydrolysis was conducted at 50 °C, pH 4.8 for 12 h. SSLP was then held at 30 °C, pH 5.0 for 144 h. Error bars represent standard deviation from the mean of three independent experiments

when the SSLP was performed, suggesting that enzymatic hydrolysis process was the rate-limiting step. Hereby, a pre-hydrolysis followed by the SSLP process was conducted to release sugars to facilitate lipid production. As depicted in Fig. 6, glucose and cello-oligosaccharides reached 11.5 g/L and 8.3 g/L, respectively, when the cellulose was pre-hydrolyzed for 12 h. Glucose was then reduced sharply to 3.0 g/L while cello-oligosaccharides remained 7.9 g/L when the SSLP was performed for 24 h. Lipid production was boosted over time and reached 9.0 g/L at 96 h, indicating that the pre-hydrolysis process could be effective in reducing the fermentation time significantly. The highest lipid titer reached 9.5 g/L, suggesting that pre-hydrolysis process was conducive to lipid production. Similar result has been achieved for microbial lipid production from dry acid pretreated corn stover by *Rhodospiridium toruloides* with the SSLP with a pre-hydrolysis step involved [34].

Conclusions

Cellobiose was more conducive than glucose to cell growth of *L. starkeyi*, regardless of the sugar concentrations. The SSLP using *T*-cellulase with low β -glucosidase activity achieved higher cellulose conversion and lipid yield than those using Cellic® CTec2 with very high β -glucosidase activity. The glucosidase generated by *L. starkeyi* was primarily cell-bound with only a minor amount secreted to extracellular space. The SSLP achieved higher lipid production when a pre-hydrolysis process was involved. This work provides valuable information for the design of a more cost-effective lignocellulose-to-lipid route.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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