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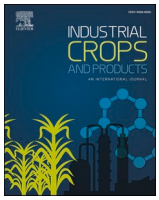

Continuous simultaneous saccharification and co-fermentation (SSCF) for cellulosic L-lactic acid production

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ABSTRACT

Continuous fermentation is preferred in industrial bioprocesses by its steady-state operation mode and the significant reduction of switch operation of vessels emptying, cleaning, and sterilizing. General biorefinery fermentation of lignocellulose is conducted in the mode of simultaneous saccharification and co-fermentation (SSCF) thus the continuous operation does not stand well because of the mismatch between the optimal temperatures of cellulase enzymes and microbial cells. This study challenged the continuous SSCF operation for production of high chiral purity L-lactic acid based on the dry biorefinery processing platform. An engineered thermophilic L-lactic acid bacterium *Pediococcus acidilactici* with complete non-glucose assimilation capacity and antibacterial activity with a nearly perfect match of temperature (42–50 °C) and pH (5.5) with that of cellulase enzyme used (50 °C, pH 4.8). This microbial cell factory allows the enzymatic hydrolysis and fermentation to be conducted simultaneously in one-pot bioreactor and provides the basis for establishing a valid continuous SSCF operation. The continuous SSCF of the biodetoxified wheat straw was established in cascade bioreactors and the performances of L-lactic acid titer, yield, and productivity reached 107.5 ± 1.1 g/L, 0.29 ± 0.01 g/g DM, and 2.69 ± 0.03 g/L/h, respectively. The chiral purity of the cellulosic L-lactic acid reached 99.5 %. The low residual sugar concentration and high-optical purity L-lactic acid product in broth facilitate the subsequent purification of L-lactic acid as the monomer for cyclic lactide or direct PLA synthesis. This multi-stage continuous SSCF technology overcomes the inherent barriers of the batch SSCF operation and provides a prototype for future industrial cellulosic lactic production.

1. Introduction

Simultaneous saccharification and co-fermentation (SSCF) is the most efficient operation for lignocellulose bioconversion by simultaneously conducting enzymatic hydrolysis and fermentation in one single reactor to lessen the sugars inhibition on cellulase enzymes (Hahn-Hagerdal et al., 2006; Lamichhane et al., 2021). The major challenge for SSCF is the mismatch of optimal temperatures between cellulase enzymes (~50 °C) and microbial cell factories (30–37 °C) (Robak and Balcerek, 2018). Therefore, a substantial compromise has to be made by splitting the SSCF into two steps of pre-hydrolysis at the higher temperature of cellulase enzymes and the consequent SSCF at the lower temperature of microbes (Liu et al., 2019; Portero Barahona et al., 2020).

Continuous fermentation is the preferred operation mode in industrial bioprocessing by its steady-state operation mode and the significant reduction of the switch operation of vessels emptying, cleaning, and sterilizing (Brethauer et al., 2014). The manufacturing of commodity bioproducts and energy products is more viable economically and technically in continuous mode (López-Gómez et al., 2019; Villadsen, 2007). However, the general continuous biorefinery fermentation of lignocellulose does not stand well because of the severe mismatch between the optimal temperatures of cellulase enzymes and microbial cells during SSCF. No investigations were found on the continuous SSCF from lignocellulose feedstock without splitting the process into two sub-steps (pre-hydrolysis and consequent continuous fermentation). One relevant report is that Ahring et al. (2016) investigated a preliminary continuous fermentation using corn stover clarified hydrolysate obtained in the upstream enzymatic hydrolysis for lactic acid continuous production with less than 50 g/L, but not in the mode of simultaneous saccharification and fermentation.

Cellulosic lactic acid production provides a perfect scenario for

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continuous SSCF by its unique agreement of the temperature values between the thermophilic lactic acid bacteria (LAB) (42–50 °C) and cellulase enzymes (50 °C), as well as the pH values between the LAB strains (4.8–5.5) and the cellulase enzymes (4.8) (Abdel-Rahman et al., 2013; Samaratunga et al., 2015). These perfect matches allow efficient enzymatic hydrolysis and lactic acid fermentation to be conducted in one single reactor as the basis of continuous SSCF operation. To meet the need for industrial processing operation for cellulosic lactic acid production, however, the continuous SSCF reactor should be capable of well mixing to overcome high viscous and solids content of up to 30 % (Brethauer and Wyman, 2010; Modenbach and Nokes, 2013; Mukasekuru et al., 2018; Georgieva et al., 2008); the SSCF system should be tolerant to contamination in the more openly continuous operation in addition (Godoy et al., 2008); the engineered LAB strain should produce the high chiral purity lactic acid without residual inhibitors and sugars used for polymerization of high molecular polylactic acid (PLA) (Augustiniene et al., 2021).

This study challenged the continuous SSCF operation for production of high chiral purity L-lactic acid based on the dry biorefinery processing platform (dry acid pretreatment and biodetoxification) (Liu et al., 2015, 2018). An engineered thermophilic LAB strain, *Pediococcus acidilactici* ZY271 with the complete non-glucose assimilation pathway and antibacterial activity was used for L-lactic acid production from all lignocellulose-derived sugars (He et al., 2022; Qiu et al., 2018; Qureshi et al., 2017). The *P. acidilactici* ZY271 strain can completely and coordinately converted the total lignocellulose-derived glucose and non-glucose sugars (xylose, arabinose, mannose, and galactose) into high-titer L-lactic acid, and the L-lactic acid broth was nearly completely free of residual sugars. The helical impeller previously designed in the SSCF reactor showed excellent performances with high saccharification yield and low energy cost (Zhang et al., 2009). Here we showed the feasibility and process performance of continuous SSCF for L-lactic acid production using *Pediococcus acidilactici* ZY271 with dry acid pretreated and biodetoxified wheat straw substrate in one single bioreactor. To obtain higher key performance indicators of L-lactic acid continuous fermentation, the continuous SSCF was further established in the cascade bioreactors. This continuous SSCF system overcomes the inherent barriers of the batch SSCF operation at high solids loading and provides a prototype for future industrial cellulosic lactic production.

2. Materials and methods

2.1. Feedstocks

Wheat straw was harvested in Nanyang city, Henan province, China, from March-June 2020. The raw feedstock was coarsely chopped to remove partial dirt and stones, and then air-dried, milled by a hammer crusher through the mesh of 10 mm in diameter. The main compositions of the raw wheat straw were determined by two-step acid hydrolysis according to the NREL protocols (Sluiter et al., 2008, 2012). The main compositions of wheat straw harvested in March (wheat straw #1) include 34.31 ± 0.14 % of cellulose, 19.25 ± 0.05 % of xylan, 22.12 ± 0.01 % of lignin, and 12.00 ± 0.22 % of ash on dry base. The main compositions of wheat straw harvested in June (wheat straw #2) include 37.50 ± 0.08 % of cellulose, 22.70 ± 0.05 % of xylan, 25.41 ± 0.06 % of lignin, and 8.89 ± 0.01 % of ash on dry base. The long operation time of continuous fermentation required a large amount of feedstock and the fluctuations in the compositions of raw wheat straw at different harvested periods may lead to slight changes in fermentable sugars concentration.

2.2. Enzymes and reagents

The cellulase enzyme Cellic CTec 2.0 was purchased from Novozymes (China), Beijing, China. The filter paper activity, cellobiase activity, and protein content were determined to be 256 FPU/mL, 4653 CBU/mL, and 81 mg protein/mL, respectively (Adney and Baker, 1996; Bradford, 1976; Ghose, 1987). The α-amylase HTAA was purchased from Genencor (China), Beijing, China. The α-amylase HTAA activity is 103,900 U/mL according to the maker's instructions. Yeast extract (LP0021B) and peptone were purchased from Oxoid Co., UK. Glucose and other chemicals in the analytical grade were purchased from Sinopharm Chemical Reagent Co., Shanghai, China.

2.3. Microorganisms and medium

The biodetoxification strain *Paecilomyces variotii* FN89 (CGMCC 17665) was isolated from the contaminated colony on acid pretreated corn stover (Zhang et al., 2021). *P. variotii* FN89 was cultured on potato dextrose agar (PDA) plate and preserved on PDA plate at 4 °C for 7 days.

The engineered bacterium *Pediococcus acidilactici* ZY271 (CGMCC 13611) was applied to cellulosic L-lactic acid fermentation without sterilizing reactors, medium, and supporting facilities (Qiu et al., 2018; Qureshi et al., 2017; Yi et al., 2016). The *P. acidilactici* ZY271 seed was cultured in a simplified Man-Rogosa-Sharp (MRS) medium containing 20 g/L of glucose, 10 g/L of peptone, 10 g/L of yeast extract, 5 g/L of sodium acetate, 2 g/L of diammonium hydrogen citrate, 2 g/L of K_2HPO_4, 0.58 g/L of $MgSO_4$0.7 H_2O, and 0.25 g/L of $MnSO_4{\cdot}H_2O$. The fermentation medium for cellulosic L-lactic acid continuous SSCF contained 10 g/L of peptone, 15 g/L of yeast extract, 2 g/L of diammonium hydrogen citrate, and 0.25 g/L of $MnSO_4{\cdot}H_2O$.

2.4. Pretreatment and biodetoxification

Sulfuric acid was used as the catalyst of dry acid pretreatment with the dosage of 4.0–4.8 % of dry matter according to the base pH approach method (Han and Bao, 2018). The pretreatment was carried out according to the previously described protocols (Liu et al., 2018). Briefly, 1200 g of wheat straw or corn stover (dry base) and 500–600 g of acid solution (adjusted based on the moisture content of the feedstock, and finally equivalent to the dry solid weight to the acid liquid weight of 2:1) were co-currently fed into the 20-L reactor with a helical impeller then maintained at 175 °C for 5 min under mild mixing by a helical stirrer. The pretreated wheat straw was in solid particles form and discharged from the bottom part of the reactor. The main compositions of the pretreated wheat straw #1 included 329.9 ± 2.9 mg cellulose, 11.1 ± 0.6 mg xylan, 42.7 ± 1.1 mg glucose, 115.2 ± 0.2 mg xylose, 21.7 ± 0.2 mg acetic acid, 6.0 ± 0.1 mg HMF, and 2.8 ± 0.1 mg furfural per gram of dry matter. The main compositions of the pretreated wheat straw #2 included 327.8 ± 0.2 mg cellulose, 11.5 ± 0.1 mg xylan, 32.3 ± 0.8 mg glucose, 147.7 ± 2.6 mg xylose, 21.3 ± 0.3 mg acetic acid, 5.8 ± 0.8 mg HMF, and 2.6 ± 0.7 mg furfural per gram of dry matter.

The pretreated wheat was neutralized to pH value of 4.8–5.5 by adding 20 % (w/w) of $Ca(OH)_2$ solution, and then disk milled to remove the residual long fibers. The pretreated wheat straw was aerobically biodetoxified by *P. variotii* FN89 in a 15-L bioreactor to remove the inhibitors. *P. variotii* FN89 has the ability to degrade most lignocellulose-derived inhibitors prior to fermentable sugars (Zhang et al., 2021). *P. variotii* FN89 was firstly cultured on PDA plate for 4 days at 37 °C. The spores on PDA plate were washed by 0.05 % (w/w) Tween 80 solution and then inoculated onto the pretreated wheat straw with the concentration of ~10^6 spores/g wheat straw, and statically maintained at 37 °C for 3 days as the biodetoxification seed. The seed was then inoculated onto the pretreated straw at 10 % (w/w) mass ratio. The biodetoxification was conducted at 37 °C in a 15-L bioreactor with the aeration of 1.0 vvm until the HMF and furfural in the pretreated wheat straw were not detected by HPLC (36–48 h). The residual acetic acid in biodetoxified wheat straw can be used for lactic acid fermentation nutrients. The total fermentable sugar loss during the biodetoxification was about 3 % (Zhang et al., 2021). The biodetoxified wheat straw was stored in disposable polyethylene plastic bags (50 cm × 60 cm in size and 25 μm in thickness) before being used for fermentation.

2.5. Single-stage continuous L-lactic acid SSCF in one reactor

The L-lactic acid continuous SSCF operation was tested in one single bioreactor. One vial of *P. acidilactici* ZY271 was inoculated into 20 mL of simplified MRS medium in a 100 mL flask and cultured at 42 °C for 12 h. Then the cells were transferred into 200 mL of the simplified MRS medium in a 500 mL flask and cultured at 42 °C for 8 h as the lactic acid fermentation seed. Glucoamylase was added into the seed culture broth at 1 % (v/v) to prevent cell flocculation (Liu et al., 2015).

The total mass of fermentation broth was set at 2200 g. The fermentation was conducted in a 5-L bioreactor equipped with the helical impeller at 42–50 °C, 150 rpm, 30 % (w/w) solids loading with the inoculation ratio of 20 % (v/w). The fermentation pH was maintained at 5.5 by automatic regulation with 25 % (w/w) of $Ca(OH)_2$ slurry. The fermentation broth was discharged at the ratio of 10–30 % (w/w) by a vacuum pump after the glucose concentration was below ~20 g/L. The materials including biodetoxified wheat straw solids, water, cellulase enzyme, and nutrients were then fed in batches, which are in balance with the mass of the discharged fermentation broth. The discharging and feeding operations were repeated every 12 h until the L-lactic acid titer reached stable or decreased continuously. It is worth noting that the flow addition of 25 % of (w/w) $Ca(OH)_2$ solution would lead to an increase in the total mass of the fermentation broth, therefore, the weight of feeding water should deduct the weight of $Ca(OH)_2$ solution.

2.6. Multi-stage continuous L-lactic acid SSCF in cascade reactors

The overall diagram of the multi-stage continuous SSCF was illustrated in Fig. 1. The raw wheat straw feedstock was pretreated and biodetoxified, and fed into the multi-stage continuous SSCF in cascade reactor series with a temporary storage tank. The multi-stage SSCF system consisted of three 5-L cascade reactors (F1, F2, F3) equipped with the helical ribbon stirrer. A fourth reactor (F4) parallel to the first one (F1) was supplemented for accelerating the overall process to a steady state. The continuous SSCF was performed in the following steps:

Step 1: The fermentation broth in F1 was periodically discharged at the mass ratio of 20–30 % (w/w) by a vacuum pump after the glucose concentration was consumed below 20 g/L. The materials including the pretreated and biodetoxified wheat straw solids, cellulase enzyme, nutrients, and water were periodically fed immediately after the discharge. The mass of feeding materials and the discharging broth were equal to keep the fermentation in steady state. The discharging and feeding operations were repeated every 8–12 h.

Step 2: The broth discharged from F1 was directly fed into F2 without sterilization, then the broth was periodically discharged from F2 at the mass ratio of 20–30 % (w/w) every 8–12 h.

Step 3: The broth discharged from F2 was directly fed into F3 without sterilization until the total mass of fermentation broth of F3 reached 2200 g.

Step 4: The broth was periodically discharged from F3 at the mass ratio of 20–30 % (w/w) every 8–12 h as the product. The same mass of fermentation broth was discharged and transferred sequentially from F2 to F3, and then from F1 to F2. Finally, the materials including biodetoxified wheat straw solids, cellulase enzyme, nutrients, and water were fed into F1.

Step 5: The continuous SSCF was ended when the fermentation reached a steady state.

The detailed operations of F4, which is parallel to F1, are as follows:

Step 1: The fermentation and inoculation were carried out in F4 following the same operations as in F1.

Step 2: When the fermentation broth was transferred from F1 to F2 for the first time, the fermentation broth in F4 was simultaneously discharged and transferred to F2 at the mass ratio of 20–30 % (w/w). No feeding operation was performed in F4 after the discharging operation.

Step 3: When the fermentation broth was transferred from F2 to F3 for the first time, the residual fermentation broth in F4 was simultaneously discharged and transferred to F3 at the mass ratio of 25–45 % (w/w). The fermentation of F4 was hereupon ended.

2.7. Data acquisition and analysis

The data are collected and analyzed according to the following protocols:

(1) The data at each time point shown in the figures is about the broth sample before the feeding operation.
(2) The continuous SSCF was ended when the fermentation reached a steady state. We consider the continuous fermentation has reached a steady state when the L-lactic acid titer in the broth discharged from F3 does not continuously decline and fluctuate by no more than 10 % at three consecutive time points either.

2.8. Calculations

The dilution rate (h^{-1}) was calculated according to the Eq. (1) as follows:

$$\text{Dilution rate}(h^{-1}) = \frac{D}{H} \quad (1)$$

where D is the mass ratio of discharged fermentation broth to the initial fermentation broth in the bioreactor; H (h) is the time interval of discharging operation.

The fermentation yield (g/g DM) was calculated according to the Eq. (2) as follows:

$$\text{Yield}(g/g\ DM) = \frac{([c] - [c_0]) \times V}{M} \quad (2)$$

where [c] (g/L) is the lactic acid titer in discharged fermentation broth;

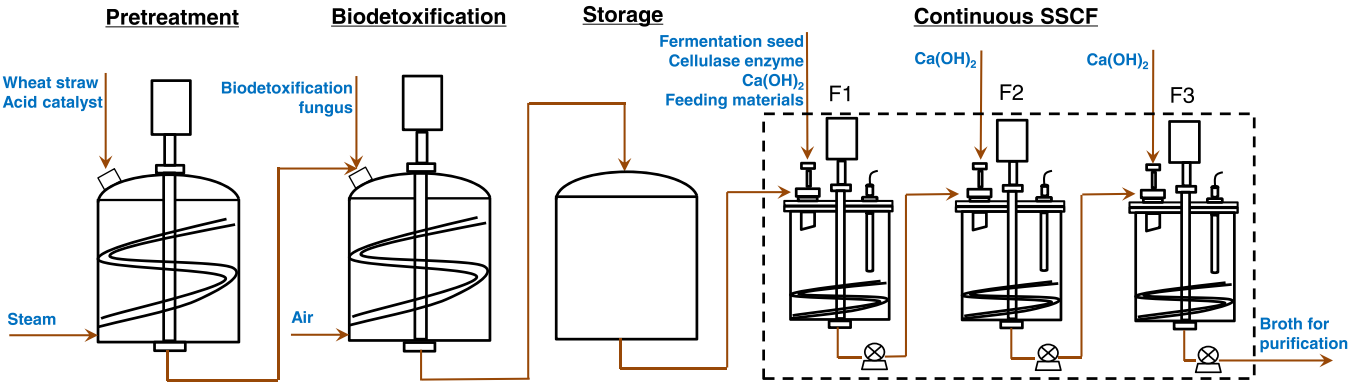

Fig. 1. Diagram of biorefinery processing chain and the multi-stage continuous SSCF for L-lactic acid production from raw wheat straw.

$[c_0]$ (g/L) is the lactic acid titer in broth after the previous round of feeding operation; V (L) is the liquid volume of the discharged fermentation broth with the density approximation of 1 g/mL; M (g) is the dry weight of feeding wheat straw. The yield was only calculated when the continuous fermentation reached a steady state.

The productivity (g/L/H) was calculated according to the Eq. (3) as follows:

$$\text{Productivity}(g/L/h) = \frac{[c] - [c_0]}{H} \quad (3)$$

where the productivity was only calculated when the continuous fermentation reached a steady state.

2.9. Analytical methods

The cell growth in the continuous SSCF process was determined by counting colony-forming units (CFU). 100 μL of 10^{-7} diluted fermentation slurry was spread on MRS Petri dishes. The colony numbers growing on the dishes at 72 °C for 48 h were counted as the CFU number. An average value of triplicate samples represented the cell growth performance. The concentrations of glucose, xylose, lactic acid, acetic acid, 5-HMF, and furfural were measured by HPLC according to the method described previously (Liu et al., 2015). The chirality of lactic acid was measured by a D-/L-lactic acid kit (Megazyme International Ireland, Bray Wicklow, Ireland) according to the manufacturer's instructions.

3. Results and discussion

3.1. Single-stage continuous SSCF in one bioreactor

The feasibility of the one-pot continuous SSCF for L-lactic acid production from lignocellulose feedstock was examined in one single bioreactor. The proposed continuous SSCF process is not purely continuous but involves pulsed solids feeding accompanied by broth discharge. The operation was conducted at 42–50 °C, and 30 % (w/w) solids loading of the acid pretreated and biodetoxified wheat straw (Fig. 2). The dilution rate was set to 0.008, 0.017, and 0.025 h^{-1}, equivalent to 0.8 %, 1.7 %, and 2.5 % (w/w) of the total broth was replaced each hour by the fresh feedstock and medium ingredients (Fig. 3). The continuous SSCF was stopped when the process reached a steady state, or the cells lost their viability.

The engineered thermotolerant lactic acid bacterium *P. acidilactici* ZY271 was applied to match the optimal temperature of cellulase enzymes (~50 °C) to ensure efficient saccharification in the one-pot SSCF. The temperature of the continuous SSCF was investigated in the range of 42, 45, 48, and 50 °C. The cell viability of *P. acidilactici* ZY271 was represented by the number of colony-forming units (CFU) because the high solids content in the hydrolysate blocked the measurement of optical density. The CFU was found to be greater than ~10^9/mL at 42 and 45 °C (Fig. 2a and b), but decreased less than ~10^9/mL at 48 °C although the L-lactic acid generation still maintained relatively constant (Fig. 2c); at 50 °C, both the cell viability and L-lactic acid generation decreased (Fig. 2d). The L-lactic acid production at 42 and 45 °C showed the maximum L-lactic acid titer of 112.2 ± 1.0 and 104.8 ± 3.5 g/L with the yield of 0.26 ± 0.01 and 0.24 ± 0.01 g/g dry biodetoxified wheat straw, and the productivity of 0.94 ± 0.01 and 0.87 ± 0.03 g/L/h, respectively. The performance at 45 °C showed slightly smaller L-lactic acid generation, but the L-lactic acid generation was stable and the cell viability was greater than that at 42 °C.

The dilution rate determines the process productivity of continuous fermentation (Brethauer and Wyman, 2010). The lower dilution rate ensures a higher conversion rate but with a lower productivity. The dilution rate of the one-pot continuous SSCF was examined by varying the discharging/feeding ratio to the overall fermentation broth in the range of 10 %, 20 %, and 30 % (corresponding to the dilute rate of 0.008,

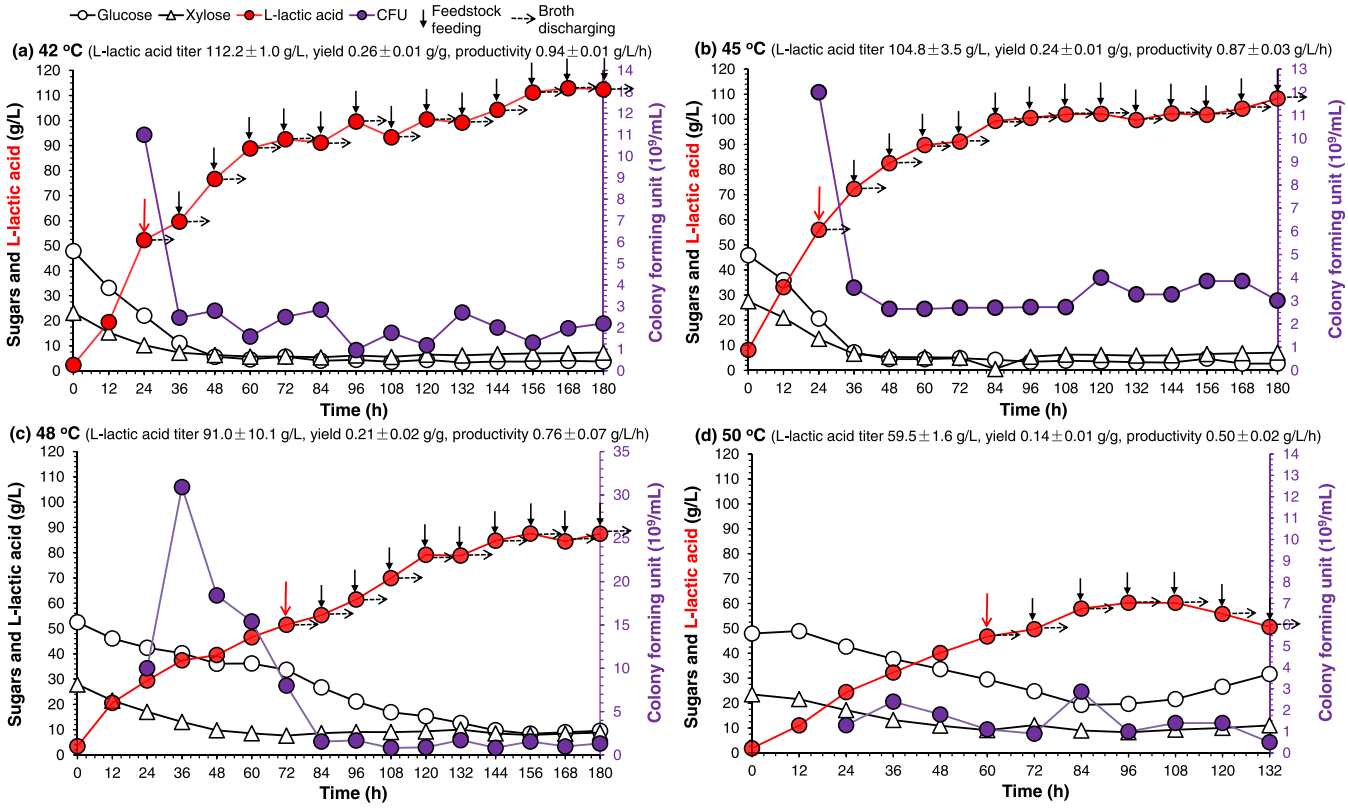

Fig. 2. Single-stage continuous SSCF at varying temperatures. (a) 42 °C; (b) 45 °C; (c) 48 °C; (d) 50 °C. Conditions: 30 % (w/w) solids loading, 150 rpm, 5 mg cellulase protein/g DM, H 12 h, D 10 % (w/w), dilution rate 0.008 h^{-1}, residual time 120.5 h. Red arrow, the feeding operation for the first time.

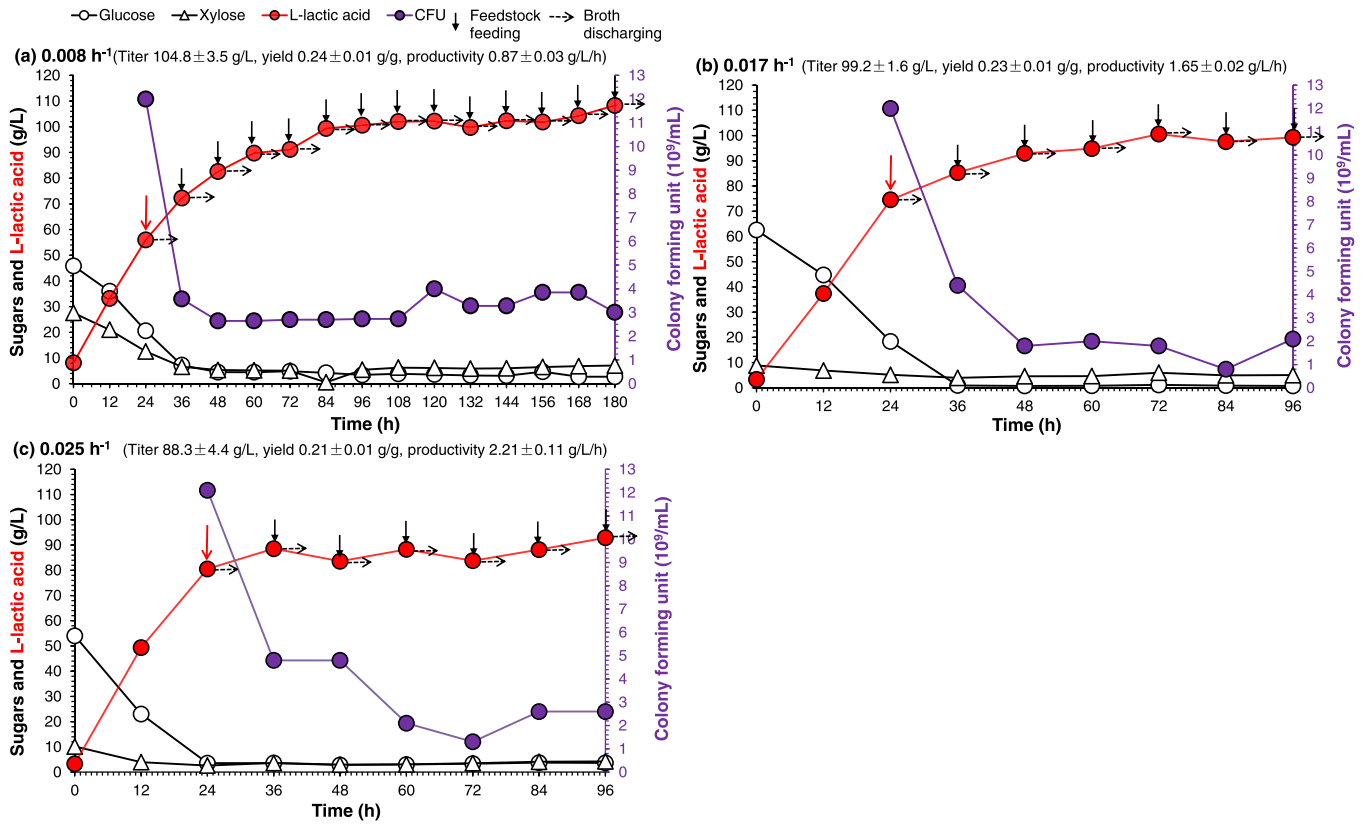

Fig. 3. Single-stage continuous SSCF with different dilution rates. (a) 0.008 h^{-1}, residual time of 120.5 h, discharging ratio of 10 % (w/w); (b) 0.017 h^{-1}, residual time of 58.8 h, discharging ratio of 20 % (w/w). (c) 0.025 h^{-1}, residual time of 40.0 h, discharging ratio of 30 % (w/w). Conditions: 30 % (w/w) solids loading, 150 rpm, 5 mg cellulase protein/g DM, 45 °C, H 12 h. Red arrow, the feeding operation for the first time.

0.017, and 0.025 h^{-1}, respectively) (Fig. 3). The L-lactic acid generation decreased and the productivity increased with the increasing dilution rate. The residual sugar accumulation and the cell viability decrease were not observed in the continuous SSCF for all the dilution rate levels. The continuous SSCF at 0.025 h^{-1} dilution rate (the average fermentation time of 40 h) resulted in the steady-state L-lactic acid generation of 88.3 ± 4.4 g/L with the yield of 0.21 ± 0.01 g/g DM and the productivity of 2.21 ± 0.11 g/L/h, indicating that the cascade reactors for the continuous SSCF were required to prolong the average fermentation time for achieving the higher L-lactic acid production.

3.2. *Multi-stage continuous SSCF in cascade bioreactors*

Multi-stage continuous fermentation in cascade bioreactors extends the residual time and correspondingly increases the product titer, conversion yield, or volumetric productivity (Brethauer and Wyman, 2010). A multi-stage cascade system composed of three 5-L bioreactors was designed and operated under the same steady-state conditions as the single-stage reactor (Fig. 4).

The solids loading content in the multi-stage continuous SSCF was examined at the dilution rate of 0.025 h^{-1} (Fig. 4a–c). At the solids loading of 30 % (w/w) (Fig. 4a), the steady-state L-lactic acid generation was 112.9 ± 2.6 g/L with the yield of 0.23 ± 0.01 g/g DM and the productivity of 2.82 ± 0.06 g/L/h. When the solids loading was reduced to 25 % and 20 % (w/w), the L-lactic acid generation was reduced to 107.5 ± 1.1 and 82.1 ± 2.1 g/L; the yield was increased to 0.29 ± 0.01 and 0.33 ± 0.01 g/g DM; and the productivity was reduced to 2.69 ± 0.03 and 2.05 ± 0.05 g/L/h, respectively (Fig. 4b and c). A trade-off between L-lactic acid production rate and yield was made to the moderate solids loading of 25 % (w/w) for multi-stage continuous SSCF (Mode II, Fig. 4b).

The dilution rate was further increased from 0.025 to 0.037 h^{-1} by raising the discharging/feeding ratio to the overall broth from 20 % to 30 % (w/w, Figs. 4b and 4d). The time to reach a steady state was shortened to 64 h and the L-lactic acid productivity increased by 26 %. However, the lactic acid titer declined with the increasing residual sugars in the first reactor (F1, Fig. 4d), indicating the dilution rate of 0.037 h^{-1} was too high to reach the steady state in the three-cascade continuous fermentation.

In summary, the multi-stage continuous SSCF in the three-cascade bioreactors significantly improved the key performance indicators of L-lactic acid production compared to the single-stage continuous SSCF in one single bioreactor. The L-lactic acid titer, yield, and productivity of multi-stage continuous SSCF **(Mode II)** increased by 21.7 %, 38.1 %, and 21.7 %, compared to those of the single-stage continuous SSCF (Figs. 4b and 3c). Furthermore, no bacterial contaminations were observed during the long period of the multi-stage continuous SSCF, owing to the antibacterial ability of *P. acidilactici* ZY271 (Qureshi et al., 2017). The chiral purity of the L-lactic acid product was greater than 99.5 %. The low residual sugar concentration and high optical purity of L-lactic acid products facilitate the subsequent purification of L-lactic acid as the monomer for cyclic lactide or direct PLA synthesis (He et al., 2022).

0.025 h^{-1}, residual time 120.0 h; (b) Mode II: solids 25 % (w/w), D 20 % (w/w), H 8 h, dilution rate 0.025 h^{-1}, residual time 120.0 h; (c) Mode III: solids 20 % (w/w), D 20 % (w/w), H 8 h, dilution rate 0.025 h^{-1}, residual time 120.0 h; (d) Mode Ⅳ: solids 25 % (w/w), D 30 % (w/w), H 8 h, dilution rate 0.037 h^{-1}, residual time 106.7 h. Conditions: 150 rpm, 5 mg protein/g DM, 45 °C. Red arrow, the first-time feeding operation in F1.

3.3. *Overall mass balance of multi-stage continuous SSCF process*

The overall mass balance of the multi-stage continuous SSCF for production of L-lactic acid was calculated from the start of virgin wheat

(a) Mode I: Solids 30% (w/w), dilution rate 0.025 h^{-1}; L-lactic acid titer 112.9±2.6 g/L, yield 0.23±0.01 g/g, productivity 2.82±0.06 g/L/h

(b) Mode II: Solids 25% (w/w), dilution rate 0.025 h^{-1}; L-lactic acid titer 107.5 ± 1.1 g/L, yield 0.29 ± 0.01 g/g, productivity 2.69 ± 0.03 g/L/h

(c) Mode III: Solids 20% (w/w), dilution rate 0.025 h^{-1}; L-lactic acid titer 82.1 ± 2.1 g/L, yield 0.33 ± 0.01 g/g, productivity 2.05 ± 0.05 g/L/h

(d) Mode IV: Solids 25% (w/w), dilution rate 0.037 h^{-1}; L-lactic acid titer 90.4 ± 2.2 g/L, yield 0.26 ± 0.01 g/g, productivity 3.39 ± 0.10 g/L/h

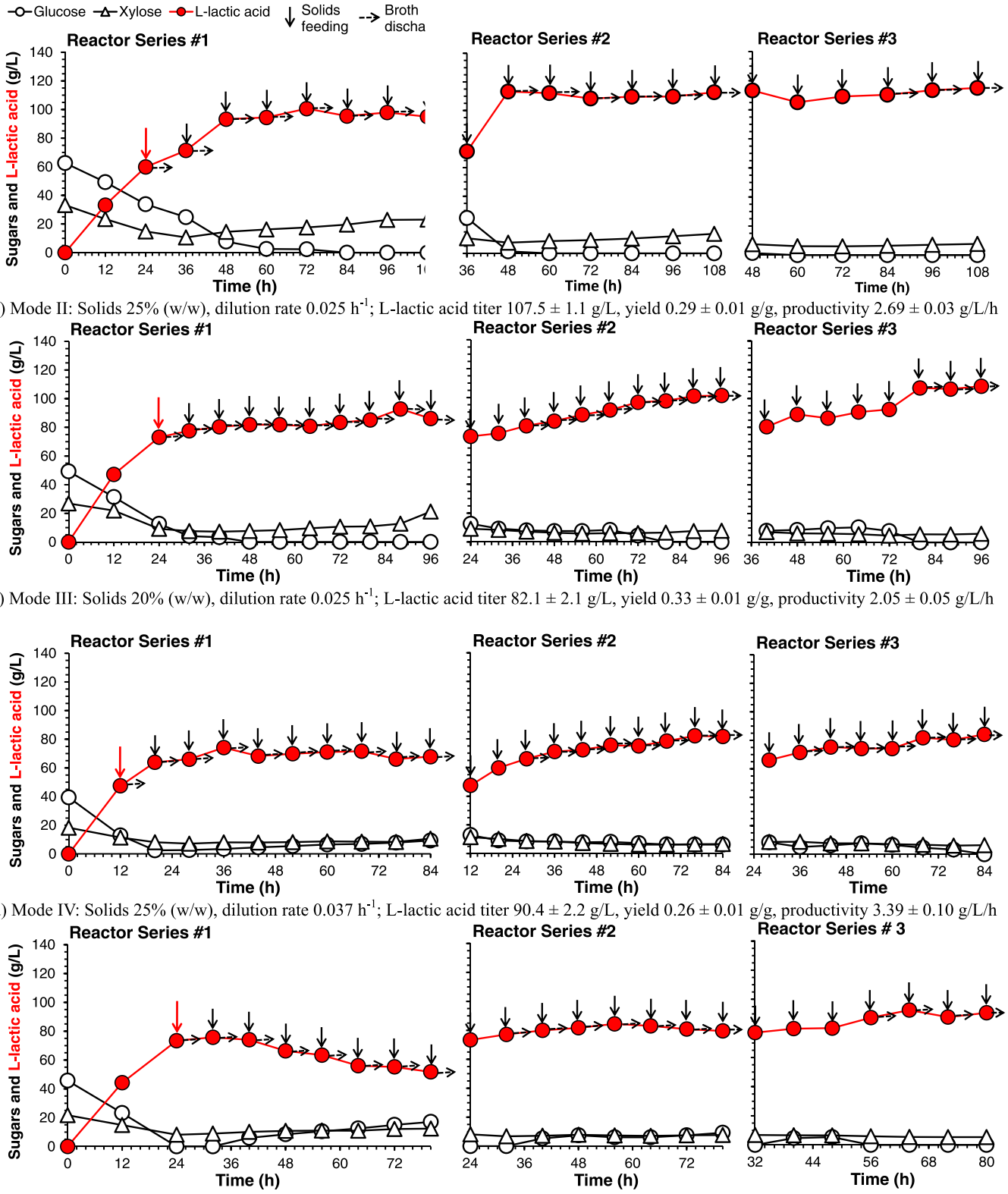

Fig. 4. Multi-stage continuous SSCF in three-cascade bioreactors. (a) Mode I: solids 30 % (w/w), D 30 %(w/w), H12h, dilution rate.

straw to the end of fermentation broth based on the experimental results of Mode II (Fig. 4b). Fig. 5 shows that 1000.0 kg of dry virgin wheat straw contains 375.0 kg of cellulose and 227.0 kg of xylan. After dry acid pretreatment, 2089.8 kg of the pretreated wheat straw is obtained by completely absorbing acid solution and condensed water (no free wastewater stream is generated). Generally, a portion (~5 %) of lignin would be converted to soluble lignin during the acid pretreatment in the presence of the aqueous phase (Humbird et al., 2011). However, the dry acid pretreatment generated no free wastewater, only a minor amount of lignin was degraded into phenolic derivatives and accumulated in the

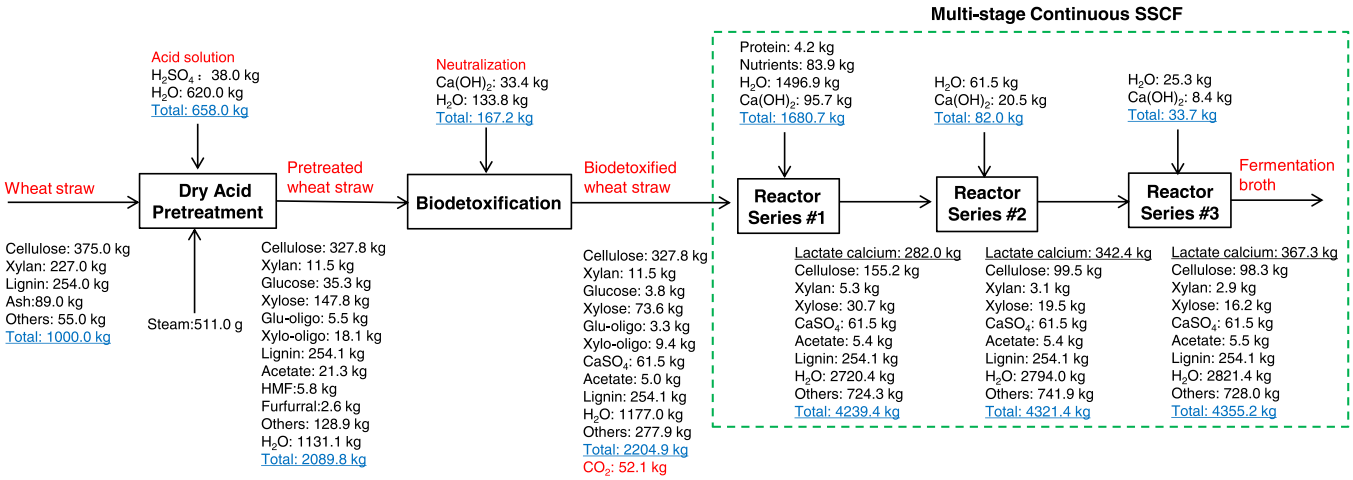

Fig. 5. Overall mass balance of cellulosic L-lactic acid continuous fermentation. Conditions of multi-stage continuous SSCF in the cascade bioreactors: solids loading 25 % (w/w), D 20 % (w/w), H 8 h, dilution rate 0.025 h^{-1}, residual time 120.0 h, 150 rpm, 5 mg cellulase protein/g DM, 45 °C. The cascade bioreactors were considered as a whole for calculation. The mass balance of continuous SSCF was calculated according to the experimental results of Mode II (Fig. 4b) at a steady state.

pretreated wheat straw. Therefore, we roughly assumed that the dry acid pretreatment will not result in the loss of insoluble lignin. Approximately 95 % of hemicellulose (xylan) is hydrolyzed into xylose and xylo-oligomers, but only 12.6 % of cellulose is converted into glucose and glu-oligomers by pretreatment. Pretreatment also generates 21.3 kg of acetic acid, 5.8 kg of HMF, and 2.6 kg of furfural. In the consequent biodetoxification step, the HMF and furfural are completely removed, and the residual acetic acid (~1/4 of the original in the pretreated wheat straw) is used as the medium component for L-lactic acid production.

The pretreated and biodetoxified wheat straw is used as the carbohydrate feedstock of the continuous SSCF for production of L-lactic acid, in which 367.3 kg of L-lactate calcium is produced, equivalently to 303.3 kg of L-lactic acid, with the yield of 0.29 g/g dry biodetoxified wheat straw. The gradual decrease in the mass of cellulose fraction and the increase in the mass of calcium lactate in the cascade bioreactors demonstrates that the multi-stage continuous SSCF process facilitates the saccharification of solid feedstock and accumulation of high titer calcium lactate compared to the single-stage continuous SSCF. The mass of unfermentable lignin fraction is consistently 254.1 kg, indicating the stability of the continuous SSCF process. The produced cellulosic L-lactate calcium is used for L-lactide synthesis following the established technical route (He et al., 2022). The L-lactic acid yield from the fermentable sugars in biodetoxified biomass is 74.3 % without considering the residual sugars. The conversion ratio of cellulose in biodetoxified biomass is only 70.0 % due to the incomplete saccharification, which is lower than that of batch SSCF (75–85 %) (Liu et al., 2015; Qiu et al., 2018).

Although the L-lactic acid production was improved by multi-stage continuous SSCF compared to that by single-stage continuous SSCF, the present L-lactic acid titer and yield of three-cascade continuous SSCF were still 10–15 % lower than those of the batch SSCF in bench-scale (Qiu et al., 2018). Further improvement for multi-stage continuous SSCF in the practical operation may consider the strict control of dilution rate and supplementation with more cascade bioreactors for a better fermentation performance.

3.4. Cases summary of cellulosic lactic acid continuous fermentation

Table 1 shows the comparisons between this study and the previous reports using various lignocellulose feedstock for lactic acid production in continuous mode. The previous reports on continuous cellulosic lactic acid production were generally conducted in lignocellulose hydrolysate supernatant solutions by separated hydrolysis and fermentation (SHF),

Table 1
Summary of cellulosic lactic acid production in continuous mode.

Feedstock	Strain	Operation	pH	Temperature (°C)	Dilution (h^{-1})	Titer (g/L)	Yield (g/g)	Optical purity (%)	Sources
Vine shoots	*L. pentosus* ATCC-8041	SHF	5.8	31	0.058	21.8	0.70[b]	nd	Bustos et al. (2007)
Rice bran	*L. rhamnosus* LA-04–01 *B. coagulans* NBRC	SHF with cell immobilization	nd	42	0.05	78.3	0.93[b]	nd	Li et al. (2015)
Corn stover	12714	SHF with cell recycling	7.0	50	0.15	92.0	0.91[b]	99.5	Ma et al. (2016)
Corn stover	*B. coagulans* AD	SHF	6.0	50	0.433	~30.0	1.09[c]	nd	Ahring et al. (2016)
Wheat straw	*B. coagulans* ATCC 23498 *L. delbrueckii* NBRC	SHF with cell recycling	6.4	50	0.357	85.4	0.86[b]	nd	Van Hecke et al. (2017)
Rice straw	3202	SHF with cell recycling	6.0	40	0.4	46.6	0.92[b]	99.5	Ma et al. (2022)
Wheat straw	*P. acidilactici* ZY271	SSCF	5.5	45	0.025	107.5	0.29[d]	99.5	This study

SHF, the lignocellulose feedstock was pretreated, enzymatically hydrolyzed, and solid/liquid separated (by centrifugation and filtration) to obtain the hydrolysate supernatant step by step, then the hydrolysate was used for continuous fermentation.
SSCF, simultaneous saccharification and co-fermentation of the pretreated and biodetoxified lignocellulose feedstock.
nd, not described.
[b] Yield of lactic acid produced (g) to consumed sugars (g).
[c] Yield of lactic acid produced (g) to biomass sugars (g).
[d] Yield of lactic acid produced (g) to biodetoxified biomass sugars (g).

instead of the direct pretreated and detoxified solid feedstock by simultaneous saccharification and co-fermentation (SSCF). The accumulation of glucose and cellobiose progressively inhibits the cellulase activity during the hydrolysis and limits the final concentrations of fermentable sugars and lactic acid (21.8–92.0 g/L) in the hydrolysates (Hahn-Hagerdal et al., 2006). The high processing cost of separation operations and the osmotic stress on the fermentation strains are also inevitable drawbacks for SHF (Robak and Balcerek, 2018; Lamichhane et al., 2021).

For the continuous SSCF using lignocellulose feedstocks, the fermentation strains need to tolerate the temperature close to the optimal temperature of cellulase enzymes (~50 °C) to ensure the efficiency of saccharification of lignocellulose biomass. The thermophilic LAB, *Bacillus coagulans*, is suitable for SSCF process due to its thermophilic growth feature and strong ability to ferment pentose for lactic acid production at 50 °C (Maas et al., 2008; Zhang et al., 2014). However, the lactic acid fermentation pH value of *B. coagulans* is between 6.0 and 7.0 (Table 1), which is higher than the optimal pH of the cellulase enzymes (~4.8). The fermentation temperature and pH of *P. acidilactici* ZY271 showed a perfect match to that of the enzymatic hydrolysis, allowing the efficient enzymatic hydrolysis and lactic acid fermentation to be conducted in one single reactor and providing the basis for continuous SSCF operation.

Cell immobilization and membrane cell recycling had been used in continuous lactic acid fermentation with relatively higher cell density and higher dilution rates. However, the addition of calcium based neutralization agents like $Ca(OH)_2$ or $CaCO_3$ and the consequent calcium lactate precipitates is expected to negatively affect the performance of the membranes (Van Hecke et al., 2017). It is practically not possible to separate the cells and the solid lignin residue in high-solids loading lignocellulose hydrolysate.

Due to the limited information in the previous publications, the lactic acid yield of the continuous lactic acid fermentation was only calculated based on the consumed sugars (0.70–0.92 g lactic acid per g consumed sugars). No residual sugars data were available and the existence of non-glucose sugars including xylose, arabinose, mannose, and galactose in chiral lactic acid broth was inevitable (Abdel-Rahman et al., 2020). The impurities such as protein, phenolics, and metal ions could be efficiently removed by the regular purification steps (He et al., 2022). However, the complete removal of residual non-glucose sugars from lactic acid broth to polymer grade monomer caused great difficulties. In this aspect, the engineered *P. acidilactici* ZY271 completely and coordinately converted the total lignocellulose-derived glucose and non-glucose sugars into high-titer L-lactic acid, and the L-lactic acid broth was nearly completely free of residual sugars. The polymerization grade L-lactide was then successfully synthesized using cellulosic L-lactic acid purified by regular methods (He et al., 2022). Therefore, the chiral cellulosic L-lactic acid obtained by continuous SSCF in this study has the potential to be used for polymerization grade L-lactide production."

4. Conclusions

This study investigated the continuous SSCF for high chiral purity cellulosic L-lactic acid production based on the dry biorefinery processing platform. The L-lactic acid fermentation temperature of 42–45 °C plus the pH agreement (4.8–5.5) with cellulase (50 °C, pH 4.8) allows for efficient enzymatic hydrolysis during the continuous SSCF. Various operation parameters were examined and the compromised optimal case was established in the cascade bioreactors with the L-lactic acid titer, yield, and productivity of 107.5 ± 1.1 g/L, 0.29 ± 0.01 g/g DM, and 2.69 ± 0.03 g/L/h. The chiral purity of the L-lactic acid product was above 99.5 %. The low residual sugar concentration and high optical purity of L-lactic acid product in broth facilitate the subsequent purification of L-lactic acid as the monomer for cyclic lactide or direct PLA synthesis. This multi-stage continuous SSCF technology overcomes the inherent barriers of the batch SSCF operation plus ease of control at a steady state and provides a prototype for future industrial cellulosic lactic production.

CRediT authorship contribution statement

Bin Zhang: Methodology, Validation, Visualization, Investigation, Data curation, Writing – review & editing. **Jing Li:** Methodology, Investigation, Data curation, Writing – original draft. **Jie Bao:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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