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Direct Succinic Acid Production from Minimally Pretreated Biomass Using Sequential Solid-State and Slurry Fermentation with Mixed Fungal Cultures

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Abstract: Conventional bio-based succinic acid production involves anaerobic bacterial fermentation of pure sugars. This study explored a new route for directly producing succinic acid from minimally-pretreated lignocellulosic biomass via a consolidated bioprocessing technology employing a mixed lignocellulolytic and acidogenic fungal co-culture. The process involved a solid-state pre-fermentation stage followed by a two-phase slurry fermentation stage. During the solid-state pre-fermentation stage, *Aspergillus niger* and *Trichoderma reesei* were co-cultured in a nitrogen-rich substrate (e.g., soybean hull) to induce cellulolytic enzyme activity. The ligninolytic fungus *Phanerochaete chrysosporium* was grown separately on carbon-rich birch wood chips to induce ligninolytic enzymes, rendering the biomass more susceptible to cellulase attack. The solid-state pre-cultures were then combined in a slurry fermentation culture to achieve simultaneous enzymatic cellulolysis and succinic acid production. This approach generated succinic acid at maximum titers of 32.43 g/L after 72 h of batch slurry fermentation (~10 g/L production), and 61.12 g/L after 36 h of addition of fresh birch wood chips at the onset of the slurry fermentation stage (~26 g/L production). Based on this result, this approach is a promising alternative to current bacterial succinic acid production due to its minimal substrate pretreatment requirements, which could reduce production costs.

Keywords: consolidated bioprocessing; fungi; solid-phase fermentation; bio-based chemicals; lignocellulose biomass; mixed cultures

1. Introduction

Succinic acid is a four-carbon 1,4-dicarboxylic acid listed in the US Department of Energy's top 12 bio-based molecules [1]. It is a "platform" chemical with wide applications in food and pharmaceuticals, surfactants, detergents, green solvents, and biodegradable plastics [2]. Succinic acid is traditionally commercially synthesized from petroleum-derived precursors; specifically, through hydrogenation of maleic acid, oxidation of 1,4-butanediol, or carbonylation of ethylene glycol [3]. An alternative pathway for bio-based succinic acid production involves anaerobic bacterial fermentation of pure sugars under CO_2-rich conditions [4,5]. Lignocellulosic biomass can be used but requires substantial pretreatment and hydrolysis to liberate fermentable sugars that can be directly utilized by succinate-producing bacteria, which can significantly add to the product cost.

This study presents a proof-of-concept for a new direct bioconversion technology for succinic acid production from minimally-pretreated non-hydrolyzed biomass. This method utilizes a mixed culture of lignocellulolytic and acidogenic fungi in a sequential solid-state and slurry fermentation process. As shown in Figure 1, the process begins with solid-state pre-cultivation of the fungal strains

on moist substrates. The goal is to initially induce and maximize cellulase, hemicellulase, and ligninase activities under static conditions, which also serves to biologically pretreat the biomass substrates in preparation for simultaneous saccharification and fermentation in the slurry fermentation stage. In the solid-state cultivation stage, nitrogen-rich (e.g., soybean hulls) and carbon-rich substrates (e.g., birch wood chips) were pre-fermented separately. The N-rich substrate was pre-fermented using a *Trichoderma reesei* and *Aspergillus niger* co-culture to induce cellulase and hemicellulase production. Prior studies have shown that *Trichoderma reesei* and *Aspergillus* spp. co-cultures are more effective for maximizing cellulolytic enzyme activities than single cultures by establishing synergistic levels of exocellulases (produced primarily by *T. reesei*) and endocellulases and β-glucosidases (produced mainly by *A. niger*) for sustained enzymatic hydrolysis and minimization of product inhibition effects [6–9]. On the other hand, the C-rich substrate was pre-fermented using *Phanerochaete chrysosporium*, which has been shown to be effective in delignifying agricultural biomass residues [10,11]. Separate solid-state pre-fermentation was done to prevent negative competitive interactions between the cellulolytic *T. reesei* and *A. niger* co-culture and the ligninolytic *P. chrysosporium*. After a sufficient cultivation time wherein optimal enzyme activities are established, the separate solid-state pre-cultures containing the fermented C-rich and N-rich substrates, fungal mycelia, and generated enzymes and other metabolites are combined (to a certain C:N ratio), submerged in a buffered medium, and incubated under continuous agitation during the slurry fermentation stage. Under the submerged two-phase fermentation condition, simultaneous biomass saccharification and fermentation of liberated biomass sugars into dicarboxylic acids such as succinic acid will occur. While few *A. niger* strains are known to produce succinic acid in low concentrations [12], typically as a minor co-product of citric acid production [13], the *T. reesei-A. niger-P. chrysosporium* triple cultures tested in this study unexpectedly favored the overproduction and accumulation of succinic acid.

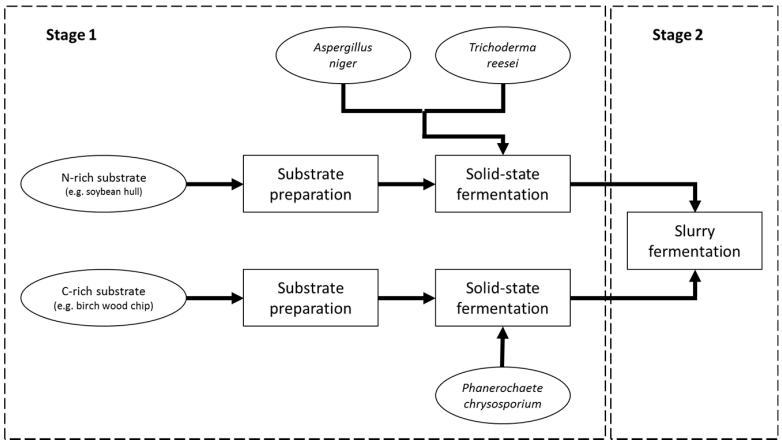


Figure 1. Sequential solid-state fermentation and slurry fermentation for direct succinic acid production from biomass using mixed fungal culture.

In this paper, we present our preliminary findings demonstrating the feasibility of this new method for direct succinic acid production from biomass substrates. Batch and fed-batch kinetic data of cellulase activity, residual sugars, and succinic acid production using *T. reesei–A. niger–P. chrysosporium* triple cultures in a sequential solid-state and slurry fermentation process are discussed.

2. Materials and Methods

2.1. Substrates

Soybean hulls and birch wood chips were used as fermentation substrates in this study. Both materials were not subjected to any physicochemical pretreatment other than size reduction and

autoclaving for sterilization purposes. Soybean hulls were obtained from Archer Daniels Midland, MI, USA. Soybean hulls typically consist of 38.4% cellulose, 10.2% hemicellulose, 2.8% lignin, and 10.7% protein [14]. Birch wood chips were provided by the Fiber and Pulp Bleaching Laboratory at Western Michigan University. Birch wood chips are typically composed of approximately 40% cellulose, 33% hemicellulose, and 20% lignin [15]. Birch wood chips were first milled to 2000-μm sizes using a Wiley laboratory mill (ED-5, Arthur H. Thomas Company, Philadelphia, PA, USA). Both soybean hulls and pre-milled birch wood chips were then further milled using a kitchen blender. The ground biomass was then screened on a stainless-steel sieve to collect substrates of approximately 500–1000 μm nominal particle diameters.

2.2. Fungi

A. niger (ATCC 15475), *T. reesei* (ATCC 56765), and *P. chrysosporium* (ATCC 48746) were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA. Fungal inocula were prepared as follows: Spores from seven-day old potato dextrose agar (PDA) slant stock cultures were suspended in sterile deionized water, poured and spread on PDA plates, and incubated at 30 °C for seven days. The spores formed on the plates were washed with sterile modified Mandel's medium containing 2 g glucose, 1.4 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.4 g $CaCl_2 \cdot 2H_2O$, 0.3 g NH_2CONH_2, 1.0 g Proteose peptone No. 2, 0.2 mL Tween-80, 5 mg $FeSO_4 \cdot 7H_2O$, 1.6 mg $MnSO_4 \cdot H_2O$, 1.4 mg $ZnSO_4 \cdot 7H_2O$, 2.0 mg $CoCl_2$ per L of deionized water 1 L [16]. To generate the fungal inocula, spore suspensions containing 1×10^6 to 1×10^7 of spores/mL were transferred to 50-mL centrifuge tubes and incubated in an incubator shaker (Innova® 43, New Brunswick Scientific Co. Inc., Enfield, CT, USA) at 30 °C, 150 rpm for two days.

2.3. Solid-State Pre-Fermentation Stage

In the solid-state pre-fermentation stage, the fungi were grown on milled biomass substrates under static conditions. The moisture content of both substrates was adjusted to 70% (wet basis) using modified Mandel's medium. These were then sterilized using a laboratory autoclave (MLS-3780, SANYO Electric Co., Ltd., Osaka, Japan) at 121 °C for 15 min. Solid-state pre-cultures containing mixed *T. reesei* and *A. niger* in soybean hulls were prepared by adding 10% (*v*/*w*) of two-day-old inoculum cultures of *T. reesei* in a 250-mL Erlenmeyer flask containing 7.5 g (d.b., dry basis) sterilized soybean hull. *A. niger* was inoculated into the same flask one day after *T. reesei* addition to ensure adequate growth of *T. reesei* before *A. niger* addition and thus balanced cellulase and β-glucosidase activities in this co-culture. Two-day-old *P. chrysosporium* inocula (10% *w*/*v*) were grown separately in 500-mL flasks containing 22.5 g (dry basis) birch wood chips to induce ligninolytic activity. All flasks were incubated in a humidified incubator (HIS33SD, Powers Scientific Inc., Warminster, PA, USA) at 30 °C, 95% relative humidity for seven days.

2.4. Slurry Fermentation Stage

After the solid-state pre-cultivation period, the static co-cultures of *T. reesei* and *A. niger* in soybean hulls were suspended in 50.0 mL 0.05 M sodium acetate buffer (pH 4.8). The resulting mixture was mixed thoroughly for 20 min before transferring to the 500-mL baffled flask containing the *P. chrysosporium* pre-culture in birch wood chips. The mixture was then agitated to suspend all solids and fungal mycelia. The dry solids content of the combined cultures became 20% (*w*/*w*) with a birch wood chips to soybean hull mass ratio of 3:1. The flasks containing the combined pre-culture slurry were then incubated in an incubator shaker (Innova® 43, New Brunswick Scientific Co. Inc., Edison, NJ, USA) at 35 °C, 150 rpm for four days.

2.5. Analyses

Samples were collected from the liquid portion of the broth every 12 h and centrifuged at 12,000 rpm for 10 min. The supernatants were diluted with deionized water to 1:5 in 15 mL centrifuge

tubes. The diluted supernatant was analyzed for cellulase activities in terms of Filter Paper Units (FPU) according to previously published methods [17]. Briefly, FPU activity was measured by incubating 0.5 mL of diluted supernatant in 1 mL 0.05 M citrate buffer, pH 4.8, and 50 °C for 60 min and measuring the amount of reducing sugar released using the dinitrosalicylic acid (DNS) assay [18]. The absorbance was read using a UV-Vis spectrophotometer (SmartSpec™ 3000, Bio-Rad Laboratories, Inc., Hercules, CA, USA) operated at a wavelength of 540 nm with glucose as standard. Sugars (cellobiose, glucose, and xylose) and organic acids (oxalic acid, citric acid, malic acid, and succinic acid) were analyzed using an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a BioRad HPX-87H Cation Exchange column and Variable Wave Detector (VWD) for organic acids and Refractive Index (RI) detector for sugars. HPLC analysis was performed at 25 °C and wavelength of 210 nm using 0.005 M H_2SO_4 mobile phase flowing at 0.5 mL/min.

3. Results and Discussion

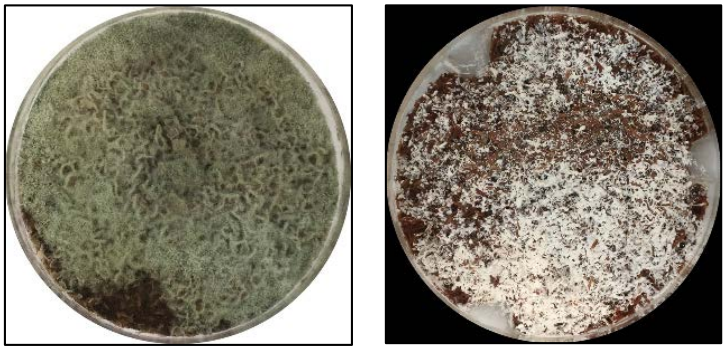
3.1. Solid-State Pre-Fermentation Stage

The initial solid-state pre-fermentation stage has a two-fold purpose: to induce fungal growth and hydrolytic enzyme activities while biologically pre-treating the biomass in preparation for direct fermentation in the slurry fermentation stage. To induce organic acid overproduction during the succeeding slurry fermentation stage, it is essential that the C-rich (i.e., birch wood chips) and N-rich (soybean hull) biomass substrates be blended to achieve an appropriately high C:N ratio. This would then necessitate inoculating all three fungal species together, simultaneously or sequentially, in the culture. However, preliminary tests showed that fungal inoculation on pre-blended soybean hull and birch wood chips substrate led to overgrowth of *P. chrysosporium* and inhibition of growth and cellulolytic activity of *T. reesei* and *A. niger*. To overcome this negative interaction effect among the fungal species in the mixed culture, it was suggested to separately pre-ferment the soybean hulls and birch wood chips before combining these substrates for the slurry fermentation stage.

Protein-rich soybean hulls are ideal substrates for inducing fungal cellulase enzyme production and were sequentially inoculated with *T. reesei* followed by *A. niger*. *A. niger* was inoculated one day after *T. reesei* to allow the proliferation of slower-growing *T. reesei*, prevent overgrowth and domination by the faster-growing *A. niger*, and establish balanced and sustained cellulase and β-glucosidase activities [8]. Figure 2a shows expansive greenish mycelia present on the surface of the soybean hull biomass with underlying black mycelia, indicating the dominant growth of *T. reesei* over *A. niger* after a one-day delay time of *A. niger* inoculation after *T. reesei*. Preliminary tests also showed that inoculating both species simultaneously resulted in *A. niger* proliferating the soybean hull surface, which reduced the chances of *T. reesei* establishing growth in a co-culture with *A. niger*.

The birch wood chips contain up to 20% (*w*/*w*) lignin [15]. Pretreatment is necessary to remove the lignin to make the cellulose and hemicellulose more accessible to enzymatic hydrolysis [19,20]. Biological pretreatment using fungi was preferred in this study over thermochemical pretreatment as it demonstrates improvement in biomass digestibility with reduced severity [21]. This study employed biological pretreatment of birch wood chip using *P. chrysosporium*, a white-rot fungi. Several studies showed that *P. chrysosporium* could significantly reduce lignin content of various agricultural residues [10,11]. Evidence of white mycelial *P. chrysosporium* growth on birch wood chips is shown in Figure 2b. However, the ligninolytic activity of the *P. chrysosporium* culture in birch wood chips was not quantified.

In addition to pre-saccharifying the substrates prior to direct acidogenic fermentation, separately pre-fermenting the soybean hulls and birch wood chips in the described manner was expected to allow growth and activation of hydrolytic enzyme activities by specialized cellulolytic and ligninolytic fungi. Hostile interactions between these fungi are prevented to allow the fungal niches to establish and sustain cellulolytic and ligninolytic activities well into the slurry fermentation stage.



(**a**) (**b**)

Figure 2. Solid-state fermentation after seven days (**a**) a mixed culture of *Trichoderma reesei* and *Aspergillus niger* on soybean hull; **(b)** *Phanerochaete chrysosporium* on birch wood chip.

3.2. Slurry Fermentation Stage

During the slurry fermentation stage, the separate solid-state pre-cultures of N-rich soybean hulls with *T. reesei* and *A. niger* and C-rich birch wood chips with *P. chrysosporium* were combined, submerged into a buffered medium, and incubated under batch conditions. Kinetic data of cellulase (filter paper-ase or FPase) activities, sugars (cellobiose, glucose, and xylose), succinic acid, and pH in a batch experiment were obtained to understand the underlying bioconversion processes and identify process variables and operating conditions that can be optimized to improve succinic acid production yields for future investigations.

Initial FPase was measured at the end of the solid-state pre-fermentation stage or immediately before transitioning to the slurry fermentation stage. FPase increased within the first 12 h of slurry fermentation and was constant between 12 and 24 h (Figure 3a). Glucose levels in the slurry culture followed this trend very closely (Figure 3b), showing a significant increase between 0 and 12 h and remained steady from 12–24 h. On the other hand, cellobiose increased after 12 h and remained fairly constant at 1 g/L for the next 24 h before it declined after 84 h. Xylose also increased to around 1 g/L after 12 h and remained constant before it declined at 72 h and was depleted after 84 h. FPase increased once more after 24 h and reached its peak at 60 h. Additionally, cellobiose doubled in concentration at 48 h which coincided with high FPase activity. However, residual glucose levels declined after 24 h and were depleted at 60 h. High FPase activity did not translate to high residual sugar present in the biomass as the generated fermentable sugars appear to have been immediately consumed for succinic acid production. At 72 h, FPase began to decline, and no residual glucose was detected. At the end of slurry fermentation, FPase went back to its original level at the start of the slurry fermentation phase, yet no further glucose production was observed. It is possible that at this period, cellulose groups susceptible to enzymatic attack may have been expended.

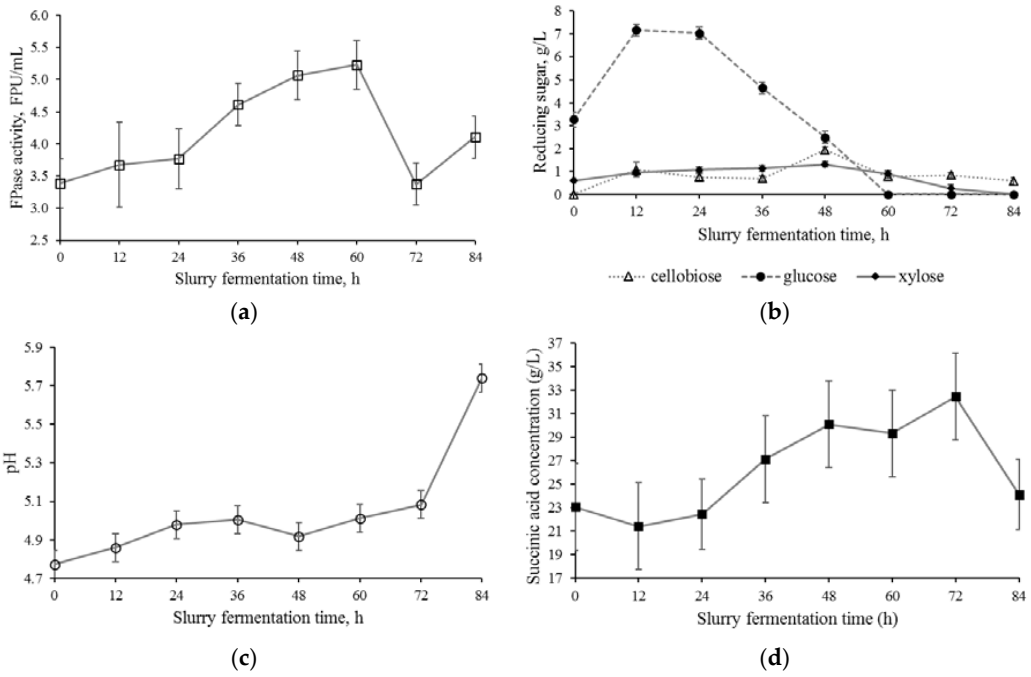


Figure 3. Time profiles of (**a**) filter paper cellulase activity, (**b**) residual sugars, (**c**) pH, and (**d**) succinic acid concentration from a mixed culture of *Trichoderma reesei*, *Aspergillus niger*, and *Phanerochaete chrysosporium* on 3:1 birch wood chip–soybean hull biomass during 84 h of slurry fermentation. Error bars represent pooled standard deviations in the data sets.

The culture pH was relatively stable during the first 72 h of slurry fermentation with values ranging from pH 4.77 to pH 5.08 (Figure 3c). For crude cellulase enzyme broth, enzymatic hydrolysis is usually conducted at pH 4.8 and 4.5 to 50 °C [22]. The pH range in the first 72 h of slurry fermentation is close to the optimum pH; however, the operating temperature in this study is lower than the reported optimum temperatures. Increasing the temperature can adversely affect the growth of the mixed fungal culture, so the culture temperature was kept close to lower recommended incubation temperatures. After 72 h, the pH increased drastically to 5.8 at 84 h, which corresponded to complete depletion of fermentable sugars and minimized filter paper cellulase activity.

As shown in Figure 3d, a substantial amount of succinic acid concentration was already produced during the solid-state pre-fermentation stage before the start of the slurry fermentation stage. At time zero, immediately after suspending the pre-cultures in the buffered media, around 23 g of succinic acid per liter of the media was detected. Unfortunately, it was not experimentally determined from which pre-culture (i.e., soybean hulls with *T. reesei* and *A. niger* vs. birch wood chips with *P. chrysosporium*) it originated. The only other organic acid that was detected was oxalic acid, which was produced at a very low concentration of 0.02 g/L. No significant increase in succinic acid levels was observed in the first 24 h (Figure 3b). Succinic acid concentrations started to increase 24 h after the maximum glucose production was reached (12 h) and continued between 24 to 48 h, after which it remained steady up to 60 h and then increased to its maximum level (32.4 g/L) at 72 h. Succinic acid production occurred concurrently with glucose consumption. The observed increase in FPase up to 60 h could indicate continuous generation of glucose through cellulolysis but this glucose was immediately consumed for acidogenesis. Beyond 72 h, succinate levels declined substantially as there was no glucose available for consumption. Other organic acids produced in minor levels during the slurry fermentation stage include citric acid and oxalic acid, which were generated at 0.89 g/L and 0.52 g/L, respectively. Both citric acid and oxalic acid were not detected after 108 h, but 0.56 g/L of malic acid was observed.

The highest succinic acid concentration obtained in the batch process under this study (32.06 g/L, after 72 h of slurry fermentation) is comparable to succinic acid levels produced by bacteria utilizing

pure fermentable sugars derived from pretreatment and hydrolysis of lignocellulosic substrates. One study obtained 23.8 g/L succinic acid using *Anaerobiospirillum succiniciproducens* from wood hydrolysate derived from steam explosion pretreatment of oak wood, and supplemented with corn steep liquor in a 32-h batch fermentation [23]. Another study produced 22.5 g/L succinic acid using *Actinobacillus succinogenes* from hemicellulose hydrolysate produced from acid pretreatment of sugarcane bagasse in a 24-h batch fermentation [24]. Both studies employed additional chemical and physicochemical pretreatment methods to obtain fermentable sugars from lignocellulosic biomass. In this study, however, direct fermentation of the lignocellulosic biomass was performed as the fungal co-culture grew directly on biomass, released enzymes that liberated fermentable sugars from the lignocellulose matrix, and converted the released fermentable sugars to succinic acid in an integrated process without severe pretreatment of the lignocellulosic biomass substrates. Succinic acid production by filamentous fungi has not yet been fully studied. High succinic acid concentration, productivity, low byproduct formation, and tolerance to low pH are some characteristics of promising fungal strains for succinic acid production [25]. Although this study mostly yielded comparable if not higher succinic acid concentrations than those reported in bacterial fermentations, its volumetric productivity of 0.45 g/L/h is still low compared to conventional bacterial succinic acid production. However, this value is higher than the reported succinic acid volumetric productivity of 0.14 g/L/h from mutant *A. niger* GCMC-7 with black strap molasses as a substrate, wherein the succinic acid is a secondary product of citric acid fermentation [13]. Bacterial succinic fermentation operates at a higher pH of 6.0 to 6.5 [23,24] while the fungal co-culture process described in this study operated at a relatively lower pH, which reduces the need for pH control at near-neutral levels.

3.3. Effect of Adding Fresh Substrate

This study also conducted preliminary runs to see the effect of introducing fresh C-rich substrate during the onset of the slurry fermentation stage on succinic acid production. It was hypothesized that adding fresh C-rich substrate could further increase the available carbon in the broth during slurry fermentation. Additional untreated milled birch wood chips (< 500 μm) were added at the start of slurry fermentation. This addition resulted in an increase in the birch wood chips-to-soybean hull ratio of 4:1 from 3:1. These fine particle birch wood chips were obtained from the undersized fraction of the milling process. All other slurry fermentation conditions such as 20% (*w*/*v*) solids loading, initial pH, temperature, and agitation speed were held constant. A maximum succinic acid concentration of 61.12 g/L was achieved after 36 h of slurry fermentation as shown in Figure 4. This amounts to a ~26 g/L succinate production relative to initial levels (i.e., transition from solid-state to slurry fermentation stage) compared to ~10 g/L production in the batch process. The addition of fresh substrate increased the succinic acid production from 40 to 77% during slurry fermentation. Additionally, the time to reach the maximum succinic acid was reduced. A maximum reducing sugar of 10.5 g/L was achieved at 24 h. When the residual reducing sugar started to decline at 36 h, the maximum succinic acid concentration was observed. At this point, the accumulated reducing sugar produced during the first 24 h of slurry fermentation was probably directly consumed to produce succinic acid. After 36 h, succinic acid and residual reducing sugar levels in the broth declined. From this preliminary result, the volumetric productivity of succinic acid production becomes 1.70 g/L·h which is 278% higher than our previous result of 0.45 g/L·h. Future studies will focus on optimizing the timing of addition of fresh substrate to further improve succinic acid production.

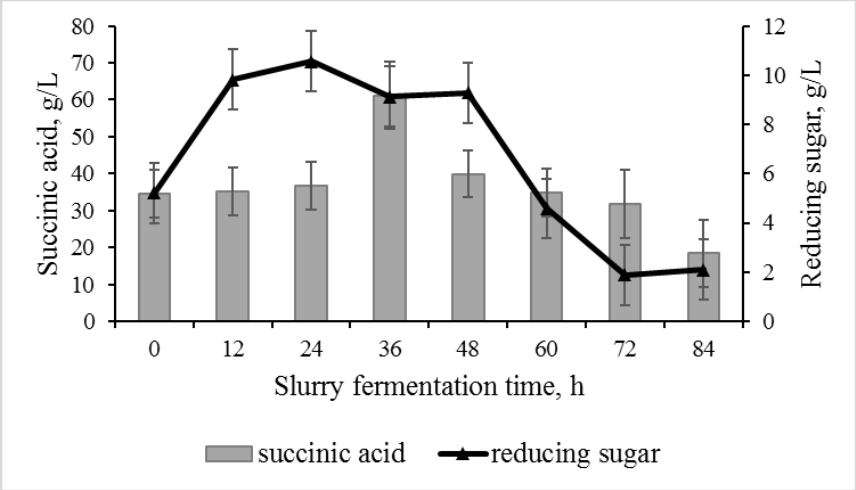


Figure 4. Time profile of succinic acid concentration and residual sugar from a mixed culture of *Trichoderma reesei*, *Aspergillus niger*, and *Phanerochaete chrysosporium* on 4:1 birch wood chip–soybean hull biomass during 84 h of slurry fermentation following the addition of fresh C-rich substrate.

4. Conclusions

This study demonstrated the feasibility of directly producing succinic acid from minimally-pretreated biomass using a new consolidated bioprocessing technique involving sequential solid-state and slurry fermentation with a mixed cellulolytic–acidogenic fungal culture. Under batch conditions, a succinic acid concentration of 32.43 g/L was achieved after 72 h of slurry fermentation, corresponding to ~10 g/L succinic acid production. An overall succinic acid yield of 13.0 g per 100 g dry substrate was also obtained. When the system was converted to fed-batch, wherein fresh substrate was introduced at the onset of the slurry fermentation stage, a succinic acid concentration of 61.12 g/L was reported, corresponding to ~26 g/L succinic acid production. This high succinic acid concentration corresponds to the volumetric productivity of 1.70 g/L·h and a yield of 24.45 g succinic acid per 100 g substrate. The next challenge is to increase the volumetric productivity of succinic acid production using a fungal co-culture for it to be competitive with bacterial succinic acid and to further minimize the cost of production. Factors that may affect fungal growth and succinic acid production such as pH, solids loading, fungal spore loading, fungal species combination, carbon substrate concentration, and others need to be investigated in future studies. Optimizing the consolidated bioprocessing and shifting to fed-batch fermentation could potentially lead to an increase in succinic acid concentration and volumetric productivity.

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