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Effect of Acetic Acid on Succinic Acid Production of Fungal Co-Culture in Slurry Fermentation

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The effect of initial acetic acid concentration on succinic acid production from biologically pretreated birchwood chip-soybean hull mixture using co-culture of *Aspergillus niger*, *Phanerochaete chrysosporium*, and *Trichoderma reesei* in a slurry fermentation set-up was investigated. Acetic acid was supplied in various initial concentration: 1.65 g/L, 3.75 g/L, and 5.85 g/L. Low initial acetic acid resulted in decline of succinic acid production which coincided with declining acetic acid concentration. On the other hand, both medium and high initial acetic acid sustained higher succinic acid production with nearly constant acetic acid concentration throughout the five-day slurry fermentation. The residual sugar concentration was also observed as this was indirectly affected by the presence of acetic acid in the mixture. The presence of acetic acid in the fermentation media plays a crucial role in the production of succinic acid from a non-conventional source, the fungal co-culture.

1. Introduction

Succinic acid is a four-1,4 dicarboxylic acid that has a variety of applications in food, pharmaceuticals, surfactants, green solvents, and as a precursor chemical for various industrially important polymers (Zeikus et al., 1999). Succinic acid together with two other diacids: fumaric and malic acids, are listed in the U.S. Department of Energy's top 12 promising bio-based chemicals (Werpy and Petersen, 2004). Succinic acid is traditionally produced from petroleum products via partial hydrogenation of maleic anhydride (Cornils and Lappe, 2000), but efforts were made to successfully commercialize succinic acid production from the sustainable route of microbial fermentation (Song and Lee, 2006). As of 2013, 38 kilotons or 49% of succinic acid produced came are from bio-based production route (E4tech, RE-CORD, and WUR, 2015).
Natural producers of succinic acid such as rumen bacteria *Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens*, and *Mannheimia succiniciproducens* were well-studied. The main substrate of bacterial fermentation are sugars derived from energy-rich crops. A cheaper alternative, which do not compete with human food consumption, is lignocellulosic biomass which can be directly utilized by filamentous fungi. This route of succinic acid production via fungal fermentation is not thoroughly explored.
It was reported that succinic acid is a secondary product at low concentrations by some *Aspergillus* spp. (Bercovitz et al., 1990; Ikram-ul et al., 2004). In the preliminary study of Alcantara and colleagues (2017), succinic acid was a primary product of the two-step fermentation of mixed birchwood chip-soybean hull substrate using the mixed fungal culture of *Aspergillus niger, Trichoderma reesei*, and *Phanerochaete chrysosporium*. This alternative route of microbial succinic acid production via a two-step fermentation process involves (1) a solid-state fermentation (SSF) pre-culturing stage followed by (2) a slurry slurry fermentation stage. In the SSF, the cellulolytic fungi *A. niger* and *T. reesei* were co-cultured on nitrogen-rich soybean hull. Co-culturing of *A. niger* and *T. reesei* has been reported to produce the optimal endoglucanase to β-glucosidase ratio that significantly improved cellulolytic activity (Dhillon et al., 2011; Fang et al., 2013; Gutierrez-Correa et al., 1999; Lu et al., 2013). On the other hand, *P. chyrsosporium, which* has shown its capability to degrade the lignin component of plant biomass (Shi et al., 2008; Zhang et al., 2011), was grown on lignin-rich birch wood chips. The removal or modification of lignin makes cellulose more accessible to enzymatic hydrolysis (McMillan, 1994; Mosier et al., 2005). Unpublished preliminary experiments attempted to

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co-culture the three fungal strains but were unsuccessful. The ligninolytic fungi, *Phanerochaete chrysosporium* tended to inhibit the growth of the other two when the three fungal strains were inoculated on the same day. Because of this, *P. chrysosporium* was grown on separate carbon-rich milled birchwood chip. On the other hand, both the *Aspergillus niger* and *Trichoderma reesei* were grown on milled soybean hull. *T. reesei* has shown to produce higher glucanase activity than β-glucosidase (Dashtban et al., 2009, Duff et al., 1987; Khan et al., 1989). On the other hand, *A. niger* produced higher β-glucosidase activity (Dashtban et al., 2009). Co-culturing *A. niger* and *T. reesei* was reported to produce higher cellulolytic activities in solid-state fermentation than in monoculture experiments (Dhillon et al., 2011; Fang et al., 2013; Gutierrez-Correa et al., 1999; Lu et al., 2013). However, it was observed that inoculating *A. niger* and *T. reesei* within the same day produced unfavorable results as the *A. niger* grow more aggressively than the *T. reesei*. To address this issue, one day time-delay inoculation technique was employed. Time-delay strategy has shown to improve cellulolytic enzyme activity of *A. niger*-*T. reesei* co-culture (Fang et al., 2013).

This study employed two-step fermentation strategy: solid-state fermentation pre-culturing stage followed by slurry fermentation stage. In the solid-state fermentation pre-culturing stage, the fungal co-cultures were grown on fixed bed of substrate in the absence of free water. This stage served dual purpose: to initiate growth of fungi and to produce lignocellulolytic enzyme necessary to degrade the biomass components into fermentable sugars. Studies have shown that solid-state fermentation has higher cellulolytic enzyme activity compared to submerged fermentation (Hölker et al., 2004; Pandey et al., 1999). The second stage, the slurry fermentation stage is characterized by the distribution of the solid substrate in the liquid phase (Glover and Kunz, 2003). The hypothesis is that sugars liberated from the biomass mixture through the synergistic cellulolytic activity of *A. niger* and *T. reesei* during the solid-state pre-culturing stage and early slurry fermentation stage were simultaneously converted into a mixed organic acid product by the acidogenic *A. niger* with succinic acid as the primary product. This study investigated the effect of various initial acetic acid concentration, one of the driving factors in fungal succinic production in the slurry fermentation stage of the two-step direct fermentation of lignocellulosic biomass.

2. Materials and Methods

2.1 Substrate and basal medium preparation

The substrates used in this study were birchwood chips (BWC) and soybean hull (SBH) (both collected in Michigan, USA). The substrates were separately air-dried to less than 10 % moisture and milled to particle size range of 500 to 1,000 μm. The milled substrates were then separately collected in a resealable polyethylene storage bag and stored in 4 °C refrigerator.

The basal medium formulated by Mandel and Weber (1969) with modifications by Tangnu et al. (1981) was prepared by dissolving 1.4 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.4 g $CaCl_0 \cdot 2H_2O$, 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 $CoCl_2$ in 1 L deionized water.

2.2 Fungal strains and inoculum preparation

The fungal strains used in this study were *Aspergillus niger* Y-78 (ATCC 15475), *Phanerochaete chrysosporium* A-381 (ATCC 48746), and *Trichoderma reesei* RUT-C30 (ATCC 56765). The fungi were cultured on potato dextrose agar (PDA) slants incubated for seven days at 25 °C. The spores in the fully sporulated slants were collected by scraping the spores and suspending them in the basal medium. The collected spores were poured on PDA plates and incubated for another seven days at 25 °C. The week-old cultures were again suspended in basal medium and transferred in a 250 mL Erlenmeyer flask. The spore count was adjusted to ~ 1 x 10^7 spore/mL by diluting it with the basal medium supplemented with 2 % w/v glucose. The spore suspensions were placed in an incubator shaker (Innova® 43, New Brunswick Scientific Co. Inc., NJ, USA) at 30 °C and 150 rpm for two days to generate a viable seed culture to inoculate the substrates.

2.3 Solid-state fermentation

Five grams and 15 g (both dry basis) of milled SBH and BWC, respectively, were placed in separate 250 mL Erlenmeyer flasks. The moisture content of both substrates was adjusted to 70 % (w/w) using the basal medium. The wet substrates were then autoclaved at 121 °C for 15 min. Five milliliters of the two-day-old *T. reesei* was inoculated on the sterilized SBH. After 24 h, 5 mL of *A. niger* was added on the same sterilized SBH initially inoculated with *T. reesei* culture. On the other hand, 5 mL of the two-day-old *P. chrysosporium* was inoculated on sterilized milled BWC. All flasks for SSF were incubated in a humidified incubator (HIS33SD, Powers Scientific Inc., PA, USA) kept at 30 °C and 95% relative humidity for seven days. Chemical

nor physicochemical pretreatment was not employed in the mixed biomass substrate as the fungal culture produced its own enzymes to degrade the biomass substrate into fermentable sugars.

2.4 Slurry fermentation

Acetic acid was prepared at various concentrations(1.65 g/L, 3.75 g/L, and 5.85 g/L) by adding glacial acetic acid to deionized water and the pH was adjusted to 4.80 by adding 0.1 N NaOH solution. The resulting acetate buffers were sterilized and cooled to room temperature. The seven-day-old *A. niger-T. reesei* co-culture on SBH and *P. chrysosporium* on BWC were combined in a 250 mL Erlenmeyer flask by rinsing the fermented biomass with sterilized acetate buffer. The solids content of the mixture was adjusted to 15% (w/w) by addition of sterilized acetate buffer. The mixtures were placed in an incubator shaker (Innova® 43, New Brunswick Scientific Co. Inc., NJ, USA) at 250 rpm for 20 minutes to thoroughly mix the components resulting to a slurry-type mixture. The agitation speed was finally reduced to 150 rpm and the resulting mixtures were incubated for five days at 30 °C.

2.5 Quantitative analyses

Samples (1.0 mL) were withdrawn from the liquid portion of the fermentation broth every 24 hours for five days. The samples were collected in a 1.5 mL Eppendorf tube and were centrifuged using microcentrifuge (5415D, Brinkman Instrument Inc., NY, USA) at 12,000 rpm for 10 minutes to separate the supernatant from the solids. The supernatants were diluted to 1:3 with deionized water and were analyzed for organic acids and residual sugar content using Agilent 1100 Series HPLC (Agilent Technologies, CA, USA) equipped with BioRad HPX-87H Cation Exchange column and Variable Wave Detector (VWD) for organic acids and Refractive Index (RI) detector for reducing sugars. Analyses were performed at 30 °C, 0.005 M H_2SO_4 mobile phase, UV absorbance at 210 nm, at a flow rate of 0.5 mL/min. The standards used were analytical grade (Sigma Aldrich, MO, USA) acetic, citric, malic, and succinic for organic acids and cellobiose, glucose, and xylose for residual sugars.

3. Results and Discussion

3.1 Effect of initial acetic acid concentration on succinic acid yield

The initial concentration of acetic acid in the slurry broth was identified as one of the crucial factors in fungal succinic acid production. Fencyl and Leopold (1958) reported the inhibitory effect of acetic acid to the succinate anion uptake of *A. niger*. In their study, inhibition was observed when the concentration of undissociated acetic acid was above 2.5 mM (~1.5 g/L). Unpublished preliminary experiments showed higher succinic acid yield when the initial acetic acid concentration was 3.75 g/L. On the other hand, at 2.0 g/L acetic acid, the succinic acid yield was depleted during the five-day slurry fermentation phase. With these results, the study set three initial acetic acid concentrations: 1.65, 3.75, and 5.85 g/L. Very high acetic acid concentration, 5% (52.2 g/L) was not considered in this study as acetic acid was reported to inhibit growth of some *Aspergillus* species (Hassan et al., 2015). The highest initial acetic acid concentration used in this study was 5.85 g/L which was 10 folds lower than the acetic acid used by Hassan and colleagues.

Figure 1 shows that the initial actual acetic acid concentration is higher than the supplemented acetic acid. Small amount of acetic acid was produced during the solid-state fermentation pre-culturing stage. In addition, other organic acids such as malic and oxalic acids were produced in minute quantities. For both middle and high-level initial acetic acid concentrations, the level of acetic acid in the fermentation broth stayed constant throughout the five-day slurry fermentation stage. On the other hand, acetic acid was depleted on the third day of slurry fermentation for low-level initial acetic acid concentration.

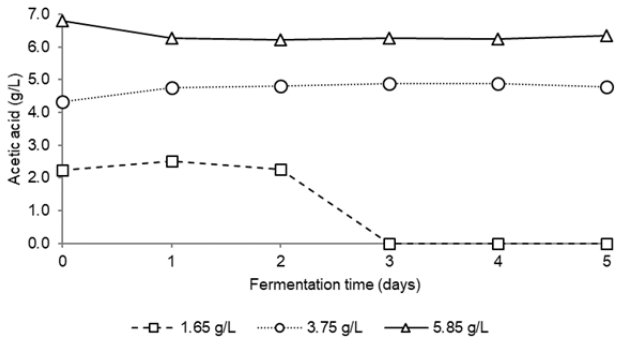

Figure 1: Acetic acid profile at different initial acetic acid concentration.

There was an increase in succinic acid yield for all treatments during the first 24 hours of slurry fermentation stage as shown in Figure 2. Afterwards, succinic acid yield slightly increased for both middle and high-level initial acetic acid concentration. For these two treatments, the amount of acetic acid was above the threshold of ~1.5 g/L. It is hypothesized that the high amount of acetic acid in the fermentation broth prevented the succinic acid uptake of the fungal co-culture. Succinic acid yield was increased by 150 % during the slurry fermentation stage for both middle and high-level initial acetic acid concentrations. For low-level initial acetic acid concentration, there was an increase after 24 hours of slurry fermentation comparable to that of middle and high-level. However, in the second day, succinic acid yield started to decline and was eventually depleted on the third day of the slurry fermentation stage. During the first two days of low-level run, acetic acid concentration remained at 2 g/L. This low-level of acetic acid concentration may have promoted succinate anion uptake of the fungal co-culture as succinic acid yield began to decline on the second day. Both acetic and succinic acid were absent on the third day of slurry fermentation phase. Since the acetic acid concentration fell below the inhibitory threshold, the extra cellular succinic acid initially present in the fermentation broth may have been consumed by the fungal co-culture.

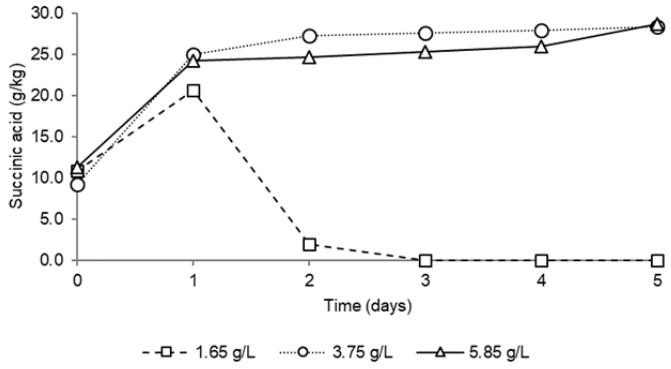

Figure 2: Succinic acid profile at different initial acetic acid concentration.

3.2 Effect of initial acetic acid concentration on residual sugar yield

In addition to the behavior of succinic acid yield in relation to the initial acetic acid concentration, the amount of residual sugar was also observed. For both medium and high-level initial acetic acid concentration, there was a steady increase in residual sugar present in the fermentation broth, as shown in Figure 3. During the slurry fermentation stage, it could be hypothesized that simultaneous enzymatic hydrolysis, which resulted to subsequent availability of fermentable sugars, and conversion of these sugars to organic acid production thru fermentation occurred. Enzymatic hydrolysis occured due to the presence of lignocellulolytic enzymes initially produced during the solid-state fermentation pre-culturing stage. Since the fermented biomass was submerged during the slurry fermentation stage with constant agitation, this possibly resulted to the increase in the rate of biomass degradation resulting to the release of fermentable sugars. It can be implied that the rate of enzymatic hydrolysis is higher than the rate of sugar consumption for microbial growth and metabolism. This could explain the steady accumulation of sugars for middle and high-level initial acetic acid concentration. There was a slight increase in succinic acid yield during this period. The presence of high amount of sugars could possibly inhibit further production of succinic acid. On the other hand, lower amount residual sugar was observed for low-level initial acetic acid run. The amount of residual sugars eventually depleted on the third day of the slurry fermentation stage. On the third day, both the acetic acid and succinic acid were absent. Acetic acid served as a buffering agent during the slurry fermentation stage. The weak strength of the buffer due to low amount of acetic acid present could possibly affected the rate enzymatic hydrolysis as other products were also simultaneously produced. The rate of cellulase and β-glucosidase activities were dependent on pH, though their actual quantities were not measured yet in this part of study. The absence of organic acids could have possibly resulted in the spike of pH, which drastically decreased the enzymatic activity in the fermentation broth. Lower enzyme activity can translate to lower rate of enzymatic hydrolysis. The lower rate of enzymatic hydrolysis will eventually result to lower succinic acid yield as the amount of fermentable sugars could only be just enough for cell growth and maintenance. Since enzymatic hydrolysis was sensitive to pH, an increase in pH due to lack of organic acids could deactivate the enzyme protein resulting to no liberation of fermentable sugar. The unavailability of fermentable sugar will starve the fungi co-culture that will eventually lead to cell death.

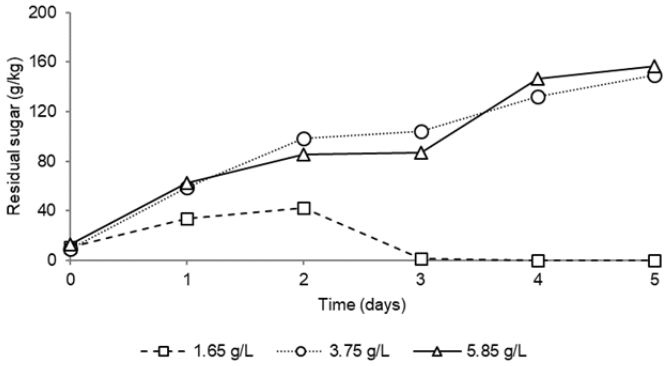

Figure 3: Reducing sugar yield profile at different initial acetic acid concentration.

4. Conclusions

Succinic acid yield was greatly affected by the presence of acetic acid. Initial acetic acid concentration of more than 1.65 g/L resulted in accumulation of succinic acid and fermentable sugars in the fermentation broth. However, it must be noted that a very high concentration of acetic acid as reported by Hassan et al. (2015) may inhibit growth of some *Aspergillus* species. On the other hand, initial acetic acid concentration of less than 1.65 g/L eventually lead to depletion of succinic acid and fermentable sugars during the course of the slurry fermentation phase. The results of this research will be useful for future optimization and kinetic studies of succinic acid production from direct fermentation of biologically pretreated-lignocellulosic biomass using fungal co-culture.

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