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Integrated production of gluconic acid and xylonic acid using dilute acid pretreated corn stover by two-stage fermentation

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ABSTRACT

A sustainable and efficient two-stage fermentation was developed to produce gluconic acid (GA) and xylonic acid (XA) from dilute acid pretreated corn stover (DA-CS) using *Gluconobacter oxydans*. Cells (6.2 g/L) were obtained after four cycles of fermentation in DA-CS enzyme hydrolysate at 50 ml scale in a shake flask. With each cycle showing complete utilization of glucose with GA productivity rate at 5.3 g/L/h. The enriched cells were then collected and used to ferment DA-CS liquid stream (generated during pretreatment containing mostly xylose) at 50 ml scale in a shake flask to produce XA at a rate of 1.9 g/L/h. Subsequently, the process was scaled up to 1 L. Both GA and XA was produced at the rate of 8.7 g/L/h and 3.7 g/L/h respectively in 36 h. From 1 kg of corn stover we were able to produce 296.2 g GA and 167.4 g XA by two stage fermentation.

1. Introduction

Agricultural residues are called lignocellulosic biomass which include wheat straw, rice straw, palm, corncobs and corn stover etc., making it an essentially inexhaustible source for environmentally friendly and biocompatible products [1]. Corn stover is one of the inedible feedstock that could be sustainably used to produce biofuels and biochemical [2]. Biochemicals such as gluconic acid (GA) and xylonic acid (XA) are extensively uses in the food, chemical, pharmaceutical, beverage, detergent, leather, construction industries due to their versatile physiological and chemical characteristics [3]. XA is considered as one of the top 30 high-value chemicals in NREL and PNNL report [4]. It is important to note that biochemical need carbon to produce them when compared to biofuels which can come from non-carbon resources (eg., hydrogen, solar). With the advancement in metabolic engineering and fermentation technology, it is possible to displace most of chemicals that are currently produced using non-renewable crude oil with sugars derived from renewable biomass [5]. Also, the biochemicals are much more valuable than biofuels and economically beneficial to produce them from lignocellulosic biomass derived sugars.

Traditionally, GA and XA can be produced by chemical, electrochemical, or enzymatic methods. Among the reported methods, microbial biotransformation has been considered to be more environmentally friendly and cost-efficiency method when compared to chemical and electrochemical method. Moreover, *Aspergillus niger* (*A. niger*) is the major strain used for producing gluconic acid and its derivatives [6]. Zhang et al., obtained 76.7 g/L GA in an aerated stirred tank reactor (ASTR) in 88 h from dry-dilute acid pretreated corn stover without detoxification using *A. niger* SIIM M276 [7]. However, the disadvantage of using *A. niger* include sensitivity to inhibitor and unable to utilize xylose. According to previous report, *Gluconobacter oxydans* (*G. oxydans*) was employed to produce GA and XA with yields of 87.6% and 31.7% respectively in 32 h. The fermentation time was reduced prevent further metabolism of GA into ketogluconic acid from corn stover [8]. As a result, complete xylose consumption was not possible.

Abbreviations: GA, gluconic acid; XA, xylonic acid; DA-CS, dilute acid pretreated corn stover; DA, dilute acid; *Gluconobacter oxydans*, *G. oxydans*; *Aspergillus niger*, *A. niger*; Glc, glucose; Xyl, xylose; XO, xylooligosaccharides; COS-SSTR, compressed oxygen supply in a sealed aerated stirred tank reactor; NREL, National Renewable Energy Laboratory; HPLC, high performance liquid chromatography; vvm, air volume/culture volume/min; ASTR, aerated stirred tank reactor.

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Bacterial strain *G. oxydans* is one of the promising industrial microbes that is used to produce aldonic acids, such as GA, 2-ketogluconic acid, and XA [9]. While carrying out mixed sugar fermentation, GA was first produced utilizing most of the glucose and then the xylose was converted to XA. However, prolonging the fermentation beyond 20–24 h, metabolizes GA into by-products such as 2-ketogluconic acid and 5-ketogluconic acid when the concentration of glucose to xylose ratio was low [10]. In order to increase GA and XA productivity, it is appropriate to carry out two stage fermentation (i.e., glucose stream to produce GA and xylose stream to produce XA).

Dilute acid pretreatment (DA) is one of the leading technology that is considered for producing fuels and chemicals. During the DA process hemicellulose is hydrolyzed to xylose and xylo-oligosaccharides (XO) rich liquid stream and insoluble cellulose rich material which could be hydrolyzed to produce glucose rich hydrolysate [11–13]. Though it is advantageous to utilize two separate sugar stream to produce two different products using microbes, degradation products produced during DA pretreatment inhibit both enzymes during hydrolysis and microbes during fermentation [14]. Detoxification methods such as over liming or separating the degradation products in the hydrolysate using ion chromatography methods are commonly used to overcome this problem [15]. Making the resistant strains by metabolic, genetic and process engineering approaches are other ways to overcome the degradation products problem.

Previous report showed that *G. oxydans* requires sorbitol-yeast extract media to activate and proliferate to generate sufficient cell density during aldonic acid fermentation [8]. However, specialized media require additional cost and will significantly increase the cost of producing aldonic acids. We have overcome this problem by using hydrolysate as media to culture *G. oxydans* instead of specialized media. Also, avoiding seed culture preparation step as demonstrated in our process will save considerable amount of time and resources.

In the present study, we have demonstrate the increased productivity and high titer of GA and XA using an integrated two stage fermentation process using dilute acid pretreated corn stover (DA-CS) liquid stream (rich in xylose) and DA-CS enzyme hydrolysate (rich in glucose). After initial optimization of the process in a shake flask at 50 ml scale, the process was further scaled up to 1L scale in a sealed aerated stirred tank reactor with compressed oxygen supply (COS-SSTR) [16]. Using two stage fermentation, we have demonstrated for the first time that high concentration of GA (296.2 g; 72.5% conversion) and XA (167.4 g; 70.4% conversion) could be produced from 1 kg of corn stover at a productivity rate of 8.7 g/L/h and 3.7 g/L/h respectively within 36 h.

2. Materials and methods

2.1. Strain and medium

G. oxydans NL71 was domesticated in lignocellulosic hydrolysate and maintained on a sorbitol agar plate which was prepared using 50 g/L sorbitol, 5 g/L yeast extract, and 20 g/L agar and stored at 4 °C. The two control medium consist of, (i) 250 mL shaker flask containing 100 g/L sorbitol, 10 g/L yeast extract and (ii) 100 g/L glucose, 5g/L yeast extract, and 20 g/L $CaCO_3$ powder. We transferred the strain from the agar plate in to sterile distilled water using a loop until OD600 reached 0.05 in a UV–vis spectrophotometer. The strain maintained in sorbitol agar plate was transferred into to a 250 mL shake flasks containing 50 mL DA-CS enzymatic hydrolysate and 20 g/L $CaCO_3$ (neutralizing agent) at 30 °C and incubated with mild agitation at 220 rpm for 20 h. After 20 h of fermentation, cells were pelleted using centrifugation and re-suspended in subsequent batch of enzymatic hydrolysate. This process was continued four time and cells pellets collected after the fourth batch of fermentation was used for producing XA using DA-CS liquid stream (generated during DA pretreatment containing mostly xylose) in shake flask same as GA producing method.

For large scale fermentation (at 1 L scale) experiment was done same as shake flask in a 3 L COS-SSTR bioreactor system at 30 °C and incubated at 500 rpm agitation in the bubbling pure oxygen (purity ≥ 99.9%) and inlet pressure of 0.02–0.05 MPa. Fermentation was done for 12 h in the case of GA production and 24 h in the case of XA production. The bioreactor was opened after every 6 h to depressurize the vessel (to remove CO_2) and enough $CaCO_3$ was added to maintain the pH. No external nutrients were supplemented to the DA-CS enzymatic hydrolysates or DA-CS liquid stream during the fermentation process except adding 20 g/L $CaCO_3$ (as a neutralizer) [17].

2.2. DA-CS liquid stream and DA-CS enzyme hydrolysate preparation

Corn stover contains 37.5%, 21.5%, and 22.1% cellulose, hemicellulose, and lignin, respectively on a dry-weight basis (w/w) as determined according to the dilute acid pretreatment method reported by the National Renewable Energy Laboratory (NREL) [18]. Corn stover was pretreated with 1.0% H_2SO_4 at a solid-liquid ratio of 1:10 at a temperature of 160 °C for 30 min. in a tumbling stainless steel reactor externally heated using electric pads. After the pretreatment process was complete, the insoluble residues was separated from soluble liquid stream using a filtration funnel. The solid pretreated biomass was washed with water until the pH reached close to neutral. The cellulose content in DA-CA solid biomass was found to be 53.7%. According our experiment results, the yield of sugar acids was lower when NaOH was used as the neutralizing agent. So the pH of the liquid stream obtained after DA-CS was adjusted to neutral pH using $Ca(OH)_2$.

The enzymatic hydrolysis was conducted in a 7.0 L stirring-tank bioreactor at 50 °C and pH 4.8 for 48 h at a loading of 20 FPU/g cellulase (Celluclast® produced by Novozymes, 1.10–1.30 g/mL, Sigma Co., Shanghai, China) at 10% total solid loading. The DA-CS enzymatic hydrolysate was concentrated by 2.9 folds until the total sugar concentration reached 100 g/L in the water bath at 60 °C.

2.3. Chemicals

Chemicals used for fermentation like Glucose, xylose, NaOH, NaOAC, GA, XA and sodium gluconate were obtained from Sigma. Calcium xylonate was obtained from Toronto Research Chemicals Inc.

2.4. Sugar and organic acid analysis

Sugars such as glucose (Glc) and xylose (Xyl); aldonic acids (GA and XA) were simultaneously determined using high performance anion-exchange chromatography coupled with pulsed amperometric detector (Dionex ICS-5000) using a CarboPacTM PA10 column. Three different solvents was used as mobile phase (a) water, (b) 200 mM NaOH and (c) 500 mM NaOAC/50 mM NaOH as mobile phase at a flow rate of 0.3 mL/min at 30 °C [19].

GA and XA yield were calculated according to the following equation:

$$\text{GA yield } (\%) = \frac{\left(C_{GA} - C_{GA_0}\right) * 0.918}{C_{Glc_0} - C_{Glc}} * 100\%$$

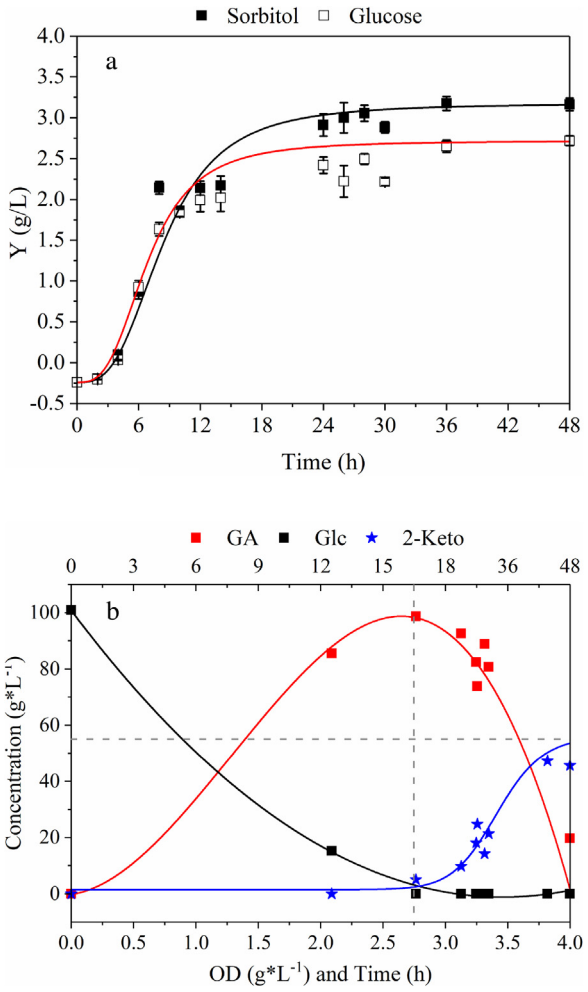

Fig. 1. Producing GA using synthetic medium. Here, a, Growth curve of *G.oxydans* using sorbitol or glucose as carbon source in shake flask and b, Kinetic of producing GA in shake flask.

$$\text{XA yield } (\%) = \frac{\left(C_{XA} - C_{XA_0}\right) * 0.904}{C_{Xyl_0} - C_{Xyl}} * 100\%$$

where C_{Glc}, C_{Xyl}, C_{GA}, and C_{XA} are the final concentrations (g/L) of Glc, Xyl, GA, and XA; C_{Glc_0}, C_{Xyl_0}, C_{GA_0}, and C_{XA_0} are the initial concentrations (g/L) of Glc, Xyl, GA and XA; the conversion factor for Glc, Xyl, GA and XA were 0.92 and 0.90, respectively. Quantification of sugars and organic acids was carried out in duplicated by using standard curves.

Organic compounds such as formic acid, acetic acid, levulinic acid, hydroxymethylfurfural (HMF), and furfural were quantitatively measured on an HPLC system (Agilent 1260) equipped with a RID detector using Aminex HPX-87H column (Bio- Rad) with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min for 55 min. Quantification of organic acids was done in duplicate in order to get consistent results.

3. Results and discussion

3.1. Fermentation using synthetic medium

G. oxydans seed culture was prepared in two different medium (sorbitol and glucose) as carbon source. The cell density increased to 2.4 ± 0.1 g/L and 2.9 ± 0.14 g/L (Fig. 1a), respectively after 24 h fermentation. During the process, GA yield was found to be 94.2% with 100% glucose utilized. The growth curve with sorbitol or glucose as carbon source is shown in Fig. 1b for 48 h. From the figure it is clear that, GA production maximized after 15 h and prolonging the fermentation up to 48 h resulted in depletion of GA and formation of 2-ketogluconic acid. At the end of 48h, 47.3 g/L of 2-ketogluconic acid was produced with a product yield of 43.5%. To maximize GA production, short fermentation time is preferred. Conventionally sorbitol is used to prepare the seed culture. However, due to additional cost associated with seed culture preparation and time taken to produce the seed culture, we completely skipped this step in our process and used DA-CS enzymatic hydrolysate as cheap alternatives.

3.2. Degradation products in hydrolysate and its effect on microbe inhibition

DA pretreatment are carried out at elevated temperature (160–190 °C) and under such conditions some of the sugar molecules are dehydrated to furfural and HMF. While, lignin is degraded into small aromatic and aliphatic molecules. These degradation products produced during DA pretreatment are found in liquid stream (rich in xylose) and are inhibitory to microbes during fermentation. The insoluble DA-CS rich stream (rich in cellulose) was washed in water to remove residual acid and degradation products and then hydrolyzed using enzymes to produce enzyme hydrolysate (rich in glucose). To further increase the sugar concentration in DA pretreated liquid stream and enzyme hydrolysate were kept in 60 °C water bath to remove water. Some of the notable degradation compounds and their concentrations (given in brackets) found in concentrated DA pretreated liquid stream include formic acid (3.1 g/L), acetic acid (12.2 g/L), levulinic acid (1.1 g/L), and HMF (0.4 g/L) were obtained. Previous studies have showed that high concentrations of inhibitors present in the DA-CS liquid stream significantly inhibited XA production by *G. oxydans.* This is due the fact that fermentation was carried out for long period and with low microbial inoculum [20]. Traditionally, detoxification such as, (i) biological [21], (ii) physical [22] and (iii) chemical methods [23] are used to remove degradation products present in DA-CS enzymatic hydrolysate. In this study, $Ca(OH)_2$ was used to adjust the pH and this results in precipitation of some toxic compounds during neutralization step [24]. According to Miao et al., nineteen selected genes in *G. oxydans* NL71 were identified as sensitive genes to the inhibitors during XA production [23]. Genetic modification methods could help to improve the tolerance of *G. oxydans* to degradation compounds.

3.3. Integrated production of aldonic acids by two-satge fermentation of hydrolysate in shake flask

GA was produced using DA-CS enzyme hydrolysate (rich in glucose), while XA was produced using DA-CS pretreated liquid stream (rich in xylose). Since significant amount of degradation products are found in DA-CS pretreated liquid stream, a high cell pitch was necessary to overcome the inhibition. In order to produce high cell density of *G.oxydans*, microbial cells were recycled by carrying out four cycles of fermentation (20 h each) using DA-CS enzyme hydrolysate in shake flask. After each cycle, the cells were pelleted by centrifugation and used for subsequent cycle. Fig. 2 shows the yield of GA at 94.7% (cycle 1), 93.1% (cycle 2), 91.8% (cycle 3), and 93.5% (cycle 4), respectively, with cell density ranging from 2.5 g/L to 6.2 g/L after four recycles every 20 h. At the end of the fourth cycle, the *G. oxydans* cell pellets were used to carry out fermentation of DA-CS pretreated liquid stream (rich in xylose) containing 21.1 g/L glucose and 89.3 g/L xylose to produce XA in shake flask for 48 h. As shown in Fig. 2, 92.2% and 88.9% yield of XA and GA were obtained after 48 h fermentation with 100% utilization of sub-

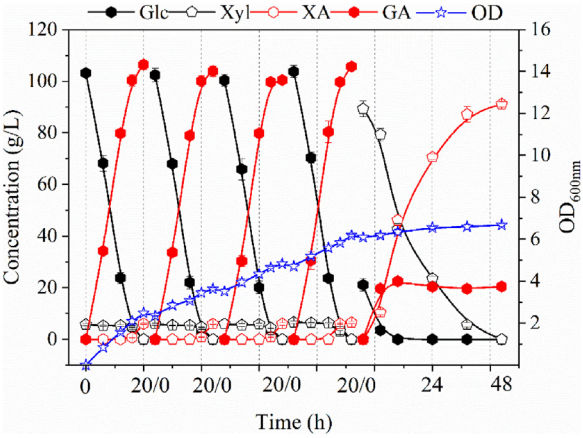

Fig. 2. Integrated production of GA and XA in shake flask using recycled cells. Cells were recycled every 20 h for 4 times in DA-CS enzymatic hydrolysate for GA production and then the recycled *G. oxydans* cells were inoculated into DA-CS liquid stream for XA production for 48 h.

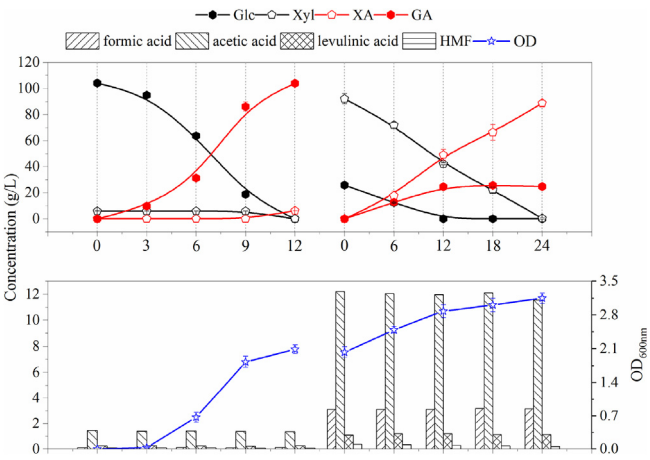

Fig. 3. Integrated production of GA and XA in COS-SSTR system. The bacteria was transfered into the concentrated DA-CS enzymatic hydrolysate for GA production for 12 h and then recycled *G. oxydans* cells were centrifuged and inoculated into the DA-CS liquid stream for XA production for 24 h in the COS-SSTR system.

strates. High yield of GA and XA production using integrated two stage fermentation used in this work demonstrate the effectiveness of this approach.

3.4. Scale-up fermentation in COS-SSTR system

Previous study on *G. oxydans* has shown that this strain harbors a group of oxido-reductase enzymes tightly bound to the bacterial membrane and linked to the cytochrome system, which depends on oxygen supply and enables directly catalyze of the substrates to several valuable chemicals [25]. To improve the bio-oxidation efficiency of fermentation, the aerated and stirred tank reactor (ASTR) was preferred. Zhang et al. obtained 132.5 g/L sodium gluconate and 15.9 g/L sodium xylonate without xylose consumption completely in a 50 L fermenter at 500 rpm with an aeration of 2.5 vvm in ASTR [8]. After considering oxygen-dependent NAD^+ regeneration during XA fermentation, Zhou et al. created a COS-SSTR system to improve XA production efficiency as well as solve the problem of foaming [12]. In this study, the two-stage fermentation was conducted in the COS-SSTR system to get higher aldonic acids productivity.

As shown in Fig. 3, the productivity of GA and XA reached 8.7 g/L/h and 3.7 g/L/h respectively in two stage fermentation in COS-SSTR, compared to 4.1 g/L/h and 0.5 g/L/h in the previous report ASTR [8]. The OD_{600} of the fermentation increased to 2.1 when concentrated DA-CS enzymatic hydrolysate was used with 95.8% yield of GA. When producing XA in DA-CS liquid stream (rich in xylose) using recycled cells, the cell growth increased from initial OD_{600} 2.0 to OD_{600} 3.2 after 24 h. These results showed that the fermentation was not inhibited by high concentrations of various inhibitors present in DA-CS liquid stream. The shortened fermentation time and higher productivity in larger scale fermenter was attributed to the sufficient and timely oxygen supply in the COS-SSTR system [26] as well as the enriched and vitalized cells, which not only accelerated the dehydrogenation reaction efficiency, but also the survivability of the strain under toxic environment.

3.5. Mass balance of integrated production of aldonic acids using two-stage fermentation

The mass balance of the whole process for producing GA and XA via two-stage fermentation was systematically analyzed. As shown in Fig. 4, 1 kg dried corn stover contained 375.1 g glucan, 215.3 g xylan, and 221.4 g lignin. After DA pretreatment was carried out at 160 °C for 30 min, 560.6 g solid pretreated corn stover (321.23 g glucan, 17.5 g xylan, and 171.9 g lignin) and 8.6 L liquid stream (30.7 g glucan, 156.5 g xylan, and 6.9 g lignin) were obtained. The pre-

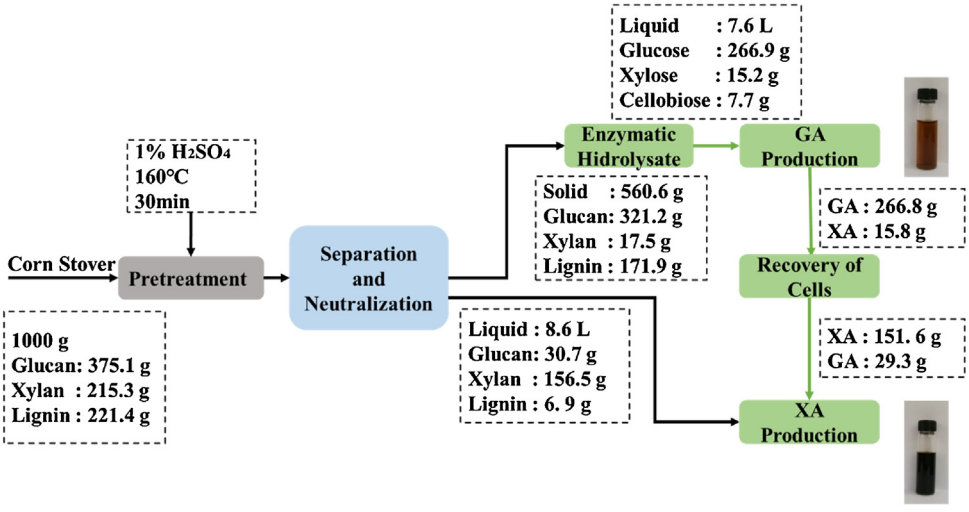

Fig. 4. Mass balance of two-stage fermentation of GA and XA using DA-CS enzymatic hydrolysate (rich in glucose) and DA-CS liquid stream (rich in xylose).

treated corn stover (solids) was enzymatically hydrolyzed using cellulase to 266.9 g glucose and 15.2 g xylose. These values correspond to the hydrolysate produced using low dosage of enzyme (20 FPU/g cellulose). During the first stage of fermentation, 266.8 g GA and 15.8 g XA were obtained from DA-CS enzymatic hydrolysate. In the second stage fermentation, using recycled *G. oxydans* cells that was obtained from first stage fermentation and DA-CS liquid stream yielded 151.6 g XA and 29.3 g GA.

The high yield of GA and XA that is produced using two-stage fermentation in this manuscript demonstrate the efficiency of the process. Further carrying out the first stage fermentation without using any seed culture and carrying out the second stage fermentation using the recycled cells generated in the first stage fermentation has help to reduce the fermentation time and processing cost. This method could be further scaled up to pre-pilot scale (100 or 1000 L) to confirm the product yield and further scale up to pilot scale (10,000 L) before commercializing the technology in a biorefinery. Economic analysis will shed more light on the scale of production that will be profitable at commercial scale.

4. Conclusions

In this study, high yield of GA and XA were produced using the recycled *G. oxydans* by a two-stage fermentation technology from DA-CS with short fermentation time using recycled cells. We have demonstrated the productivity of GA and XA in COS-SSTR was 8.7 g/L/h and 3.7 g/L/h when compared to shake flask which gave 5.3 g/L/h and 1.9 g/L/h respectively. Recycled cell produced using first stage fermentation helped to generate high cell density *G. oxydans* that was used for subsequent second stage fermentation to overcome microbial inhibition. Also, carrying out the experiments in COS-SSTR helped to provide sufficient and timely oxygen supply to *G. oxydans* which helped to increase the GA and XA yield. Finally, mass balance analysis of the entire process showed that 296.2 g GA (72.5% conversion) and 167.4 g XA (70.4% conversion) could be obtained per 1 kg dried corn stover. This study has helped to prove the concept that GA and XA could be produced in high yield at shorter period of time using recycled cells which could pave the way for commercial scale production in the near future.

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