Effect of High Solids Loading on Bacterial Contamination in Lignocellulosic Ethanol Production

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Contamination by lactic acid-producing bacteria is frequently a major challenge in ethanol processes. In this work, high solids loading was used both to keep bacterial infection under control in simultaneous saccharification and fermentation (SSF) of lignocellulosic biomass and to increase the ethanol productivity of the process. With no sterilization of the substrates, lactic acid bacteria contaminated the fermentation process with 8 and 10% suspended solids (SS) substrates, consumed both pentoses and hexoses, and produced lactic acid. However, a high solids loading of 12% SS prevented lactic acid formation, which resulted in higher ethanol yield during the SSF process. This high SS resulted in an ethanol concentration of 47.2 g/L, which satisfies the requirement for industrial lignocellulosic ethanol production.

Keywords: Ethanol; Lignocellulosic; High solids loading; Contamination; Saccharomyces cerevisiae; Lactic acid; Birch

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INTRODUCTION

Ethanol from lignocellulosic biomass, also referred to as second-generation ethanol, has received global attention because it has the potential to reduce society's dependence on fossil fuels. Lignocellulosic ethanol has numerous advantages in comparison to starch- and sugar-based ethanol from environmental and energetic points of view (Farrell *et al.* 2006; Olofsson *et al.* 2008). Various methods have been suggested to decompose the carbohydrates in lignocellulosic material, a common approach being to pretreat the material at a high temperature and low pH and then enzymatically hydrolyze it prior to fermentation. SSF (simultaneous saccharification and fermentation) combines enzymatic hydrolysis and the fermentation to ethanol in one vessel to avoid product inhibition of the enzymes.

Bacterial contamination is still a major challenge in industrial ethanol fermentations despite a lot of effort devoted to the subject (Skinner and Leathers 2004; Kádár *et al.* 2007; Bischoff *et al.* 2009; Albers *et al.* 2011; Beckner *et al.* 2011). Bacteria compete with the yeasts by consuming the sugars available for ethanol production and the nutrients needed by the yeast for growth (Skinner and Leathers 2004). As the bacteria tend to dominate the fermentation with time, inhibitory byproducts such as lactic and acetic acid are produced, which reduces the ethanol yield (Muthaiyan and Ricke 2010). It is thus of great importance to control bacterial contaminations, which otherwise increase the maintenance costs for the process (Skinner and Leathers 2004). Suggested methods for keeping bacterial contaminations under control are low pH fermentations (Kádár *et al.*

2007), use of antibiotics, and early detection methods (Muthaiyan and Ricke 2010). The use of low pH frequently fails, as some bacterial strains can thrive in low pH media (Bischoff *et al.* 2009). Antibiotics generate difficult-to-treat waste and can contribute to the development of antibiotic-resistant bacteria (Beckner *et al.* 2011). The early detection methods are laborious and offer no real solution to the problem.

Low solids loading of around 5% suspended solids have been applied on numerous informative studies in lignocellulosic ethanol processes. However, higher solid loading is required for a viable process economy (Hodge *et al.* 2008; Zhang *et al.* 2010; Modenbach and Nokes 2012). Higher solid loading constitutes a less diluted medium, which results in higher ethanol concentration (Jørgensen *et al.* 2007; Roche *et al.* 2009) and also results in less wastewater treatment (Stickel *et al.* 2009). High solids loading, however, may result in mass transfer limitations, stirring problems, and higher inhibitor concentrations compared with that of low solids loadings. Alternatively, the relatively high inhibitor concentrations may be useful as a tool to keep infectants at bay in the time-span of an SSF production cycle.

The aim of this study was to investigate the use of high solids loading to control bacterial infection during SSF of lignocellulosic biomass for ethanol production. The inhibitory effects of the fermentation media on the fermentation were studied, as well as the vitality of the yeast *S. cerevisiae* in different concentrations of suspended solids.

EXPERIMENTAL

Lignocellulosic Material

Birch chips, a hardwood lignocellulosic biomass from Swedish forests, were used. The chips were chemically pretreated with SO_2 impregnation at 18 bars and 215 °C for 5 min (SEKAB E-Technology, Sweden) and delivered as slurry. The slurry had a pH of 2.0, 16.0% suspended solids (SS), and 23.8% total solids (TS) determined according to the NREL protocol (Sluiter *et al.* 2008). The slurry was stored in a cold room at 5 °C until use. The composition of the liquid fraction of the slurry is shown in Table 1. The solid fraction of the slurry had 53.0 ± 1.4% cellulose, 38.6 ± 0.8% acid-insoluble lignin (AIL), and 5.3 ± 0.11% acid-soluble lignin (ASL), determined according to the NREL protocol (Sluiter *et al.* 2011). The total sugar in the pretreated slurry is mainly xylose (68%), and the hemicellulose fraction has been completely hydrolyzed into monomeric sugars during the pretreatment.

Table 1. Composition of the Liquid Fraction of the Birch Slurry in Undiluted Form

Component	Concentration (g/L)
Glucose	8.1
Xylose	38.1
Arabinose	2.4
Galactose	2.4
Mannose	5.1
Furfural	1.2
5-Hydroxymethyl furfural (HMF)	4.2
Acetic acid	21.3

Enzymes and Yeast Strain

Cellic® Ctec2 enzyme (Novozymes, Denmark) was used for the hydrolysis. It had an activity of 168 FPU/mL, determined according to the NREL method (Adney and Baker 2008). A commercial strain of yeast *S. cerevisiae* (Ethanol Red, Fermentis, France) was used in dry form in all the experiments. It was stored at 4 °C before use.

Simultaneous Saccharification and Fermentation (SSF)

SSF was carried out using a 2.5 L bioreactor (Minifors, Infors AG, Switzerland) with 8, 10, and 12% suspended solids using a stepwise addition of slurry. The slurry was added with different dilutions with deionized water in three steps at 0 h, 16 h, and 40 h of SSF to obtain the intended SS. The raw slurry had 16.0% SS. For the 8% SS experiment, the SS concentration was gradually increased from 3.6%, to 5.8% and then to 8.0% by the three additions, ignoring any decrease of SS by enzymatic decomposition. For the 10% SS experiment the corresponding additions raised the SS concentration from 3.6% to 6.8% and then to 10%. For the 12% experiment, the corresponding additions raised the SS concentration from 4.4% to 8.2% and then to 12%. The gradual increase in SS concentration also resulted in a gradual increase in inhibitor concentrations. In all the experiments, the first, second and third additions, were 800 mL, 500 mL and 500 mL respectively in volume resulting in a final volume of 1800 mL.

The pH of the slurry was adjusted to 5.0 using 2 M NaOH and automatically regulated during the fermentations. Deionized water was used to adjust the final fermentation volume to 1800 mL. The temperature was set at 35 °C, and an enzyme load of 21 FPU/g SS was used in all the experiments. Agitation was provided at 500 rpm, and the pH was set at 5.5. The mixture was supplemented with nutrients as 5.5 g/L yeast extract (Scharlau) and 5.5 g/L ammonium sulfate (Scharlau), and then inoculated with 6 g/L *S. cerevisiae*; 0.6 g/L silicone antifoam was also added to prevent foaming. The experiments were run in duplicate, and average values are reported. The SSF was conducted for 160 h, and samples were taken at 4-h intervals and analyzed for glucose, ethanol, and other metabolites. All experiments were run in duplicates and the average values reported. The standard deviations for lactic acid are ±26, ±4.3, and ±1.2 for the 8%, 10%, and 12% experiments, respectively.

Analytical Methods

Characterization of the slurry

Cellulose, hemicelluloses, and lignin in the solid fraction of the slurry were determined according to NREL protocols (Sluiter *et al.* 2011). The slurry was first centrifuged at 4000×g for 5 min to separate the solid and liquid fractions. The solid fraction was washed with about 40 mL deionized water several times to a neutral pH and then freeze-dried (Labconco, USA) at -52 °C until its moisture content was reduced to less than 10%. Freeze-dried samples were then hydrolyzed in two steps using 72% H_2SO_4 in a water bath at 30 °C for 60 min, followed by hydrolysis using 4% H_2SO_4 in an autoclave at 121 °C for 60 min. The 72% H_2SO_4 concentration was diluted to 4% by addition of 84.0 mL deionized water. Acid-soluble lignin (ASL) was determined using a UV spectrophotometer (Libra S60, Biochrom, England) at 283 nm and 25 L/(g cm) as the ε value. Acid-insoluble lignin (AIL) was gravimetrically determined as the residual solid after hydrolysis corrected with ash content. The ash content was determined in a muffle furnace at 575 °C overnight. Monomeric sugars contained in the hydrolysis liquid were determined by HPLC.

Sugars and metabolites analysis

The sugars and metabolic products during SSF were analyzed using an HPLC (Waters 2695, Walters Corporation, Milford, USA). A hydrogen-based column (Aminex HPX-87H, Bio-Rad, Hercules, USA) at 60 °C with 0.6 mL/min 5 mM H_2SO_4 as the eluent was used for glucose, furans, carboxylic acids, ethanol, glycerol, and lactic acid. Mannose, glucose, galactose, xylose, and arabinose were analyzed using an Aminex HPX-87P column (Bio-Rad) at 85 °C with 0.6 mL/min ultrapure water as the eluent. A UV absorbance detector (Waters 2487), operating at 210 nm, was used in series with a refractive index (RI) detector (Waters 2414).

Determination of cell vitality

Staining with a Trypan blue dye exclusion assay was used to evaluate the cell vitality. The cell suspension was mixed with Trypan blue dye, followed by visualization and counting to determine the number of stained and unstained cells (Stoddart 2011). Trypan blue solution contains 0.5% Trypan blue dissolved in 0.9% NaCl. Culture samples were taken from the fermentor at different intervals, diluted 100 times, and mixed with 100 µL of Trypan blue. A drop of the mixture was placed in a hemocytometer counting chamber using a light microscope with a 40X magnification, and cells were counted to determine the number of vital cells in the fermentation medium at different fermentation times according to the equation below:

$$\text{Number of cells/µL} = \text{number of cells in the medium square} * 250.00$$

About 20 to 30 square fields with a determined volume were counted in each observation, and the average number is reported.

RESULTS AND DISCUSSION

Lactic acid bacteria tend to survive under environmental conditions similar to the yeast *S. cerevisiae* and are the most notorious contaminants in ethanol fermentations (Skinner and Leathers 2004). High solids loading has been suggested (Jørgensen *et al.* 2007) as a means to achieve the required industrial ethanol concentration.

The presence of bacteria was confirmed by microscopic observations in all experiments. The quantification, however, was assessed indirectly by measuring the lactic acid concentration, which is arguably the most relevant output from an industrial perspective.

To investigate the influence of different solid loadings on lactic acid production caused by bacterial contamination during simultaneous saccharification and fermentation (SSF), a birch slurry was used at different suspended solids (SS) concentrations; 8%, 10%, and 12%. An enzyme load of 21 FPU/g SS was used in all the experiments, as well as the same cell density. The effect of higher solid load and thus a more inhibitory medium on the fermentation as well as the vitality of the yeast cells during fermentation was investigated.

Effect of Different Suspended Solids on Lactic Acid Production

Lactic acid was generally produced in high concentrations in the 10% and 8% SS experiments until the end of the fermentation, which lasted 160 h. At 160 h of SSF,

average concentrations of 42.6 g/L and 35.5 g/L were produced from 10% and 8% SS, respectively. In contrast, the 12% SS experiment produced a significantly lower concentration of lactic acid (Fig. 1). Only 2.9 g/L was produced during the 12% SS experiment.

The fermenting strain of *S. cerevisiae* used is a robust and suitable strain for industrial hexose fermentation. However, lactic acid bacteria are always present in almost all commercially supplied yeasts (Champagne *et al.* 2003; O'Brien 2006). Furthermore, the birch slurry was not autoclaved in order to mimic commercial processes. From a scientific perspective, this approach can be debated, but the method is highly relevant from a large-scale industrial perspective, where environments completely free of contaminants hardly exist. The significantly lower lactic acid concentration with 12% SS suggested that, with this particular substrate, there appears to be a "tipping point" between 10% and 12% SS, where the additional increase in inhibitors or stress resulted in reduced lactic acid production. However, a different medium may obviously have a different tipping point in terms of dilution and SS concentration, depending on raw material and pretreatment conditions. Thus, the less diluted slurry with 12% SS in this project is high enough to reduce the development of lactic acid-producing bacteria, while the alcohol production by the fermenting organism (*S. cerevisiae*) was proceeding. This suggests that high solids loading could be used to control bacterial infections and reduce lactic acid formation, favoring the ethanol yield. A prerequisite is the use of a robust production strain that tolerates the resulting inhibitory environment.

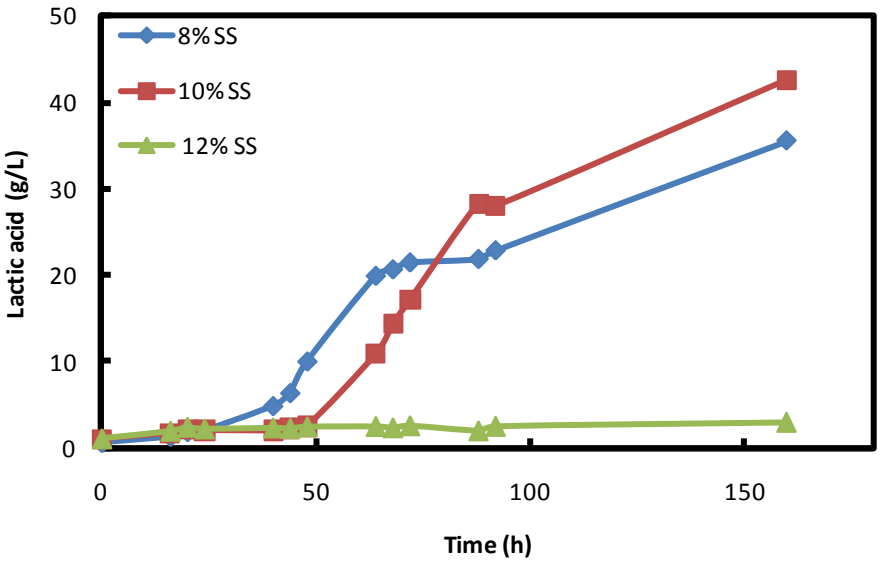

Fig. 1. Lactic acid production during SSF with different SS concentrations

The present study did not examine the effect of individual inhibitors, but it is noted that acetic acid had a concentration of 15.2 g/L in the 12% SS trial, compared to 12.5 and 9.7 g/L in the 10% and 8% SS experiments, respectively. Acetic acid has a strong pH-dependent inhibitory effect (Taherzadeh 1999), and because the yeast is more acid-tolerant than many bacteria (Matsushika *et al.* 2009), this compound may selectively target bacteria while it stimulates ethanol production by *S. cerevisiae* (Taherzadeh *et al.* 1997). Bacteria are adaptive organisms, and the whole concept of infection control by an inhibitory substrate depends on the possibility of avoiding long-term adaptation by

infectants. During SSF, the fermenting culture is regularly replaced, and the equipment can be sterilized, which prevents adaptation of bacteria.

Sugar Consumption and Ethanol Production

In spite of the inhibitory conditions during the 12% SS, the strain of *S. cerevisiae* could efficiently metabolize available glucose (Fig. 2). During the period between 48 and 160 h of fermentation, the glucose concentration slowly decreased from 4.1 to 2.5 g/L. In the same time-span, the ethanol concentration increased from 27.1 to 47.2 g/L, whereas the lactic acid concentration barely increased, from 2.4 to 2.5 g/L. Thus, the dominating processes must be the release of glucose by enzymatic decomposition of the cellulose in the solid fraction and fermentation into ethanol by *S. cerevisiae*. A certain decrease of other sugars' concentrations (mannose, galactose, xylose, and arabinose), by approximately 6 g/L, may reflect consumption of mannose and possibly also galactose by *S. cerevisiae*, as it is a hexose-consuming strain. There was a moderate increase in glycerol concentration, 1.0 g/L, and the acetic acid concentration only increased by 0.3 g/L.

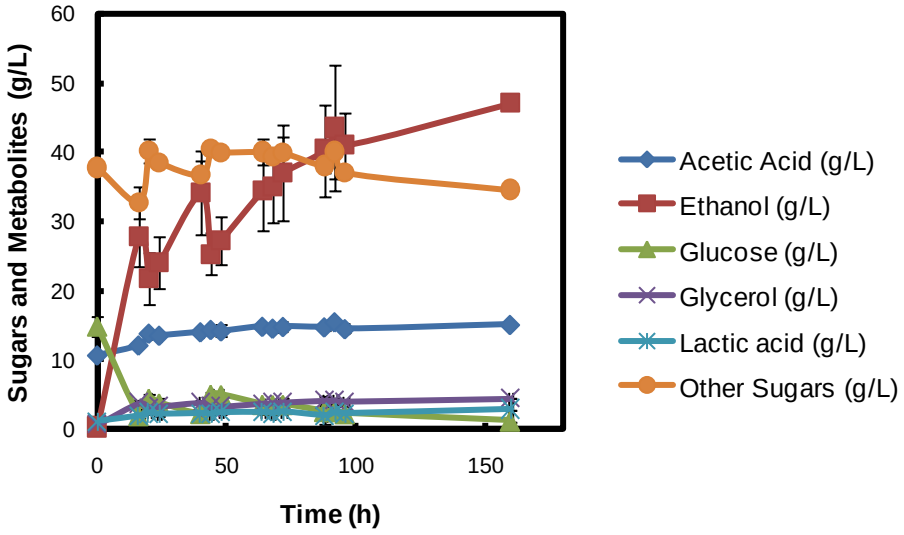

Fig. 2. Sugar and metabolite concentrations during SSF with 12% SS

A comparison with the 10% SS experiment is interesting (Fig. 3). After 48 h SSF, the ethanol concentration was 24.3 g/L and the lactic acid concentration was 2.5 g/L; a very similar development compared to the experiment with 12% SS. However, the time-span between 48 and 160 h of fermentation was very different. The glucose concentration decreased from 1.4 to 0.3 g/L, and the ethanol concentration also decreased, from 24.3 to 18.6 g/L. The lactic acid and acetic acid concentrations increased from 2.5 to 42.6 g/L and from 12.0 to 16.8 g/L, respectively. Furthermore, the concentration of other sugars decreased from 28.3 to 0.4 g/L. Figures 4a and 4b show a comparison of ethanol production and consumption of total sugars in all three experiments.

The initial concentration of other sugars in 8% and 10% SS was 30.5 g/L and 34.6 g/L, respectively. Considering a theoretical lactic acid yield of 1.0 g/g consumed sugar, the presence of 35.6 g/L and 42.6 g/L lactic acid in these respective experiments suggests that both sugars initially present in the liquid phase and glucose released from the solid phase have been fermented into lactic acid (Fig. 1). This corresponds with previous reports concerning the influence of lactic acid production on monomeric sugar

consumption during ethanol production (Stenberg *et al.* 2000). Erdei *et al.* (2010) developed an equation that can be used to estimate the amount of additional ethanol that could have been produced from hexoses if there had not been any lactic acid formation:

Mass of additional ethanol =

0.46 (*mass of lactic acid produced – mass of pentose consumed*)

The factor 0.46 g/g corresponds to 90% of the maximum theoretical ethanol yield for sugars. Applying this calculation, additional ethanol could have been produced (Table 2) during the 8% and 10% SS experiments, assuming no bacterial contamination. Although the lower SS experiment was easier to handle from a mixing and mass transfer point of view, the lower solid loading provided a more suitable environment for bacterial growth with a greater contamination risk than higher loadings during SSF. The sugar consumption by *S. cerevisiae* was reduced, whereas "infecting" organisms consumed carbohydrates and produced lactic acid and probably also acetic acid. This means that bacteria consumed not only pentoses, but also hexoses. Considering the decreased metabolic activity of *S. cerevisiae*, the yeast was exposed to the combined impact of increasing concentrations of both lactic acid and acetic acid. It is conspicuous that the onset of lactic acid production was delayed to after 48 h of fermentation. The explanation can be an adaptation phase of the bacteria in this inhibitory medium, possibly in combination with *in-situ* detoxification by the yeast. The picture was similar for the experiments with 8% SS, but with higher variation between the individual experiments.

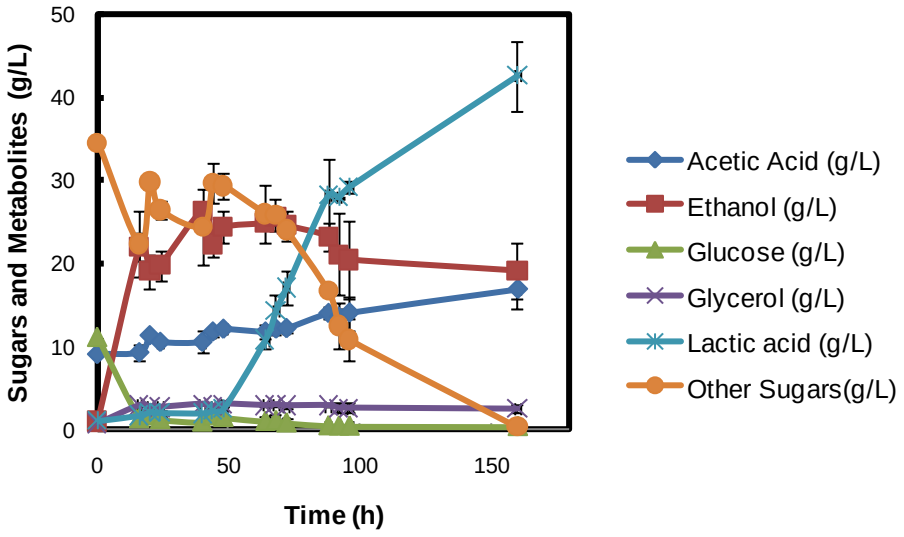

Fig. 3. Sugar and metabolite concentrations during SSF with 10% SS

Table 2. Recalculated Ethanol Assuming No Lactic Acid Formation in 8% and 10% Suspended Solids

Suspended Solids (%)	Pentose Consumed (g)	Lactic acid Produced (g)	Ethanol Produced (g)	Recalculated ethanol assuming no lactic acid formation (g)
8	46.2	57.8	38.6	44.0
10	55.5	67.4	31.0	36.5

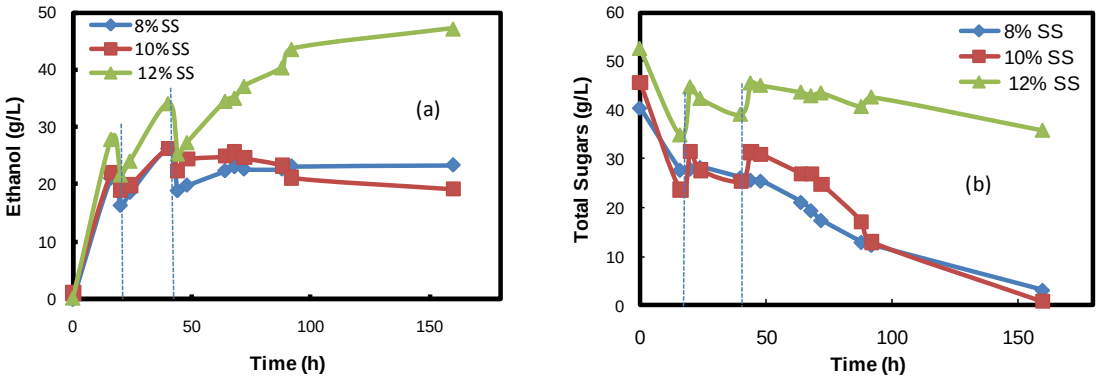

Fig. 4. Ethanol production and consumption of total sugars during SSF of different suspended solids. The dashed lines indicate the dilution effect created in the fermentor after each stepwise addition of pretreated birch slurry.

S. cerevisiae cell count in SSF

Samples were regularly collected to assess the concentration of yeast cells in the SSF experiments. The results indicate that there was a decreasing trend in yeast concentration during the fermentation in every single experiment (Fig. 5). There was approximately a 70 to 80% decrease in vitality of the cells during the first 48 h of the experiments.

The vital cell number as well as the total cells decreased sharply until they leveled out. A possible explanation is that the mechanical stress caused by the combination of agitation and solid particles grinds down the yeast cells within the first 48 h of the experiment, before the cell concentration stabilizes.

Rudolf *et al*. (2005) observed a similar trend from the colony-forming units of *S. cerevisiae* during SSF of lignocellulosic material. However, this would have caused a dramatic effect on the metabolic activity of the yeast cells, but no direct effect on the ethanol yield was observed.

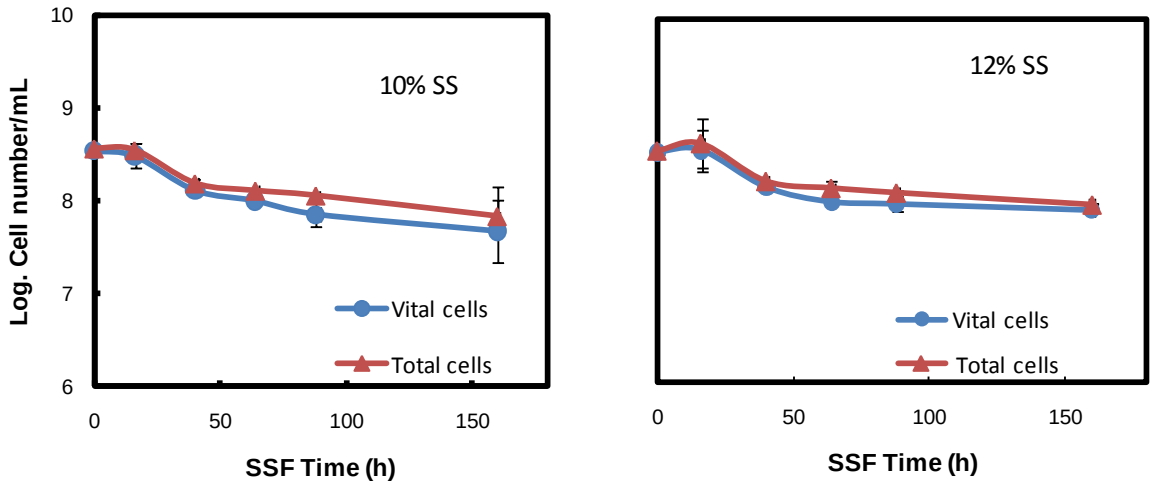

Fig. 5. Logarithm values of yeast cell number/mL versus time (h) during SSF with 10% and 12% suspended solids

CONCLUSIONS

1. A high solids loading of 12% SS reduced the production of lactic acid during simultaneous saccharification and fermentation of lignocellulosic ethanol production.
2. Ethanol productivity was increased by the use of high solids loading and an ethanol concentration of 47.2 g/L, which satisfies the requirement for industrial lignocellulosic ethanol production.
3. High solids loading prevents pentose consumption during hexose fermentation of lignocellulosic material.
4. In all experiments, the total cell concentration decreased 70 to 80% during the first 48 h of fermentation, which could indicate that the mechanical stress caused by the combination of agitation and solid particles grinds down the yeast cells within the first 48 h of the experiment, before the cell concentration stabilizes.

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