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# Ethanol Production From Dilute-Acid Softwood Hydrolysate by Co-Culture

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## Abstract

Dilute-acid softwood hydrolysate, with glucose and xylose as the dominant sugars, was fermented to ethanol by co-cultures. The strains used include *Saccharomyces cerevisiae* 2.535 (1#), *Pachysolen tannophilis* ATCC 2.1662 (2#), and recombinant *Escherichia coli* (3#) constructed in our laboratory carrying both *pdc* and *adhB* genes derived from *Zymomonas mobilis*. Before fermentation, the co-cultures were adapted for five batches. Observation under light microscope showed aggregation of adapted strains, which could possibly improve their ability to degrade inhibitors. In addition, we tried to detoxify the dilute-acid softwood hydrolysate with a combined method before fermentation. Our study showed that fermentation of detoxified hydrolysate by adapted co-culture (1# + 2#) generated an exceptionally high ethanol yield on total sugar of 0.49 g/g, corresponding to 96.1% of the maximal theoretical value after 48 h; fermentation of detoxified hydrolysate by adapted co-culture (1# + 3#) is faster (24 h) and could reach a high ethanol yield (0.45 g/g total sugar). These experiments suggest that both adaptation and detoxification significantly improve hydrolysate fermentation and ethanol production.

**Index Entries:** Dilute-acid softwood hydrolysate; co-cultures; strain adaptation; batch fermentation; ethanol fermentation.

## Introduction

Lignocellulosic biomass, a low-cost renewable resource including wood, grass, forestry waste, agricultural residues, and municipal solid

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waste, can be potentially fermented into ethanol by microbial technology that would fulfill increasing demand in fuel ethanol *(1)*. Lignocellulose-derived ethanol is an environmentally friendly liquid fuel, because exhausted carbon dioxide is taken up through a growing biomass and therefore makes no net contribution to the atmosphere *(2)*.

Lignocellulosics, containing sugars polymerized to cellulose and hemicellulose liberated when hydrolyzing the material, are subsequently fermented to ethanol by microorganisms. Dilute-acid biomass hydrolysates contain biomass degradation products inhibiting cell growth and fermentation *(3)*. A variety of biological toxins including acetic acid, degradation products of sugars such as furfural (dehydration product of pentoses) and 5-hydroxymethylfurfural (dehydration product of hexoses), and soluble aromatics from lignin (aromatic alcohols, acids, and aldehydes) are also generated during dilute-acid hydrolysis *(4)*. These toxins must be reduced or removed during fermentation for increasing ethanol production. In fact, many detoxification methods, including laccase *(5)*, ion-exchange resins *(6)*, activated carbon, boiling *(7)*, wood ash *(8)*, and treatment with alkali *(9)*, have been reported. Unfortunately, all the methods have their limitations. As each detoxification method is specific to certain types of compounds, better results can be obtained by combining two or more different methods.

Fermentation of hydrolysate has traditionally been investigated in batch processes. However, one of the major drawbacks of batch fermentation is the high initial concentration of the inhibitors in the hydrolysate, resulting in a long log phase or sometimes even failure of the fermentation *(10)*. In the present study, a combination of mixed microorganisms' adaptation and detoxification of hydrolysate were used to overcome this problem. Adaptation can help to shorten fermentation time as well as to strengthen the tolerance of the cells against inhibitors *(11,12)*. Based on the advantage of *Pachysolen tannophilis* ATCC 2.1662/recombinant *Escherichia coli* in fermenting xylose into ethanol, and the advantage of *Saccharomyces cerevisiae* in metabolizing inhibitors in low concentration, we combined and tested two groups of co-culture, *S. cerevisiae* 2.535 (1#) and *P. tannophilis* ATCC 2.1662 (2#), *S. cerevisiae* 2.535 (1#) and recombinant *E. coli* (3#) for hydrolysate fermentation in the experiments. The performance of adapted co-cultures was examined in the treated and untreated hydrolysate. Similar experiments by unadapted co-culture were used as references for evaluating the effect of adaptation. Our experimental results suggest that both adaptation and detoxification significantly improve hydrolysate fermentation and ethanol yield.

## Materials and Methods

### *Microorganism*

*S. cerevisiae* 2.535 (1#) was provided by the China General Microbiological Culture Collection Center (CGMCC). *P. tannophilis* ATCC 2.1662 (2#) was obtained from China Industrial Cultures Center (CICC). Recom-

binant *E. coli* (3#), containing both *pdc* and *adhB* genes derived from *Zymomonas mobilis* with ampicillin resistance, was constructed in our laboratory.

### *Softwood Hydrolysate*

The hydrolysate used in the experiment was provided by Department of Energy Chemical Engineering, East China University of Science and Technology, Shanghai, China. Softwood chips were immersed in 2% HCl and 0.5% $FeCl_2$ (v / v) at 170°C for 30 min. The liquid phase from the acid hydrolysis was recovered. The hydrolysate contained about 37.5 g/L of monomeric sugars, approx 75% of which was glucose. It also contained the inhibitors acetic acid (5.3 g/L), furfural (2.2 g/L), and 5-hydroxymethylfurfural. The final pH for the solution was 1.0.

### *Detoxification of Softwood Hydrolysate*

To precipitate toxic components, reduce some inhibitors, and prevent the sugars from degrading to furfural and 5-hydroxymethylfurfural, the hydrolysate was overtitrated at first with NaOH to pH 8.5 and held for 1 h at 25°C (*2*). Then the suspension was filtered and boiled at 100°C for 15 min for removing acetic acid, furfural, and some volatile fraction *(4)*. After cooling at 25°C, any loss in volume during boiling was replaced with distilled water, and then regulated to pH 5.5 for co-culture 1# and 2#, pH 7.0 for 1# and 3# with 1 *N* sulfuric acid. As opposed to the treated hydrolysate, the untreated hydrolysate was neither overtitrated to pH 8.5 nor heated. Only pH adjustment to 5.5/7.0 by NaOH was implemented.

### *Synthetic Complex Media*

The synthetic complex media was used to prepare the inoculum. The recombinant *E. coli* was cultivated in liquid Luria-Bertani (LB) medium supplemented with 15.0 g/L glucose and 60 mg/L ampicillin at pH 7.0. The medium for yeast incubation was as follows: 3 g/L tryptone, 2.5 g/L yeast extract, 2.5 g/L $KH_2PO_4$, 0.25 g/L $MgSO_4$, 0.25 g/L $CaCl_2$, and 15.0 g/L glucose at pH 5.5. The media were further sterilized by autoclaving at 121°C for 20 min.

### *Hydrolysate Media for Adaptation and Ethanol Fermentation*

For enhancing hydrolysate fermentation, both treated and untreated hydrolysate were supplemented with nutrients, including: 3 g/L tryptone, 2.5 g/L yeast extract, 2.5 g/L $KH_2PO_4$, 0.25 g/L $MgSO_4$, and 0.25 g/L $CaCl_2$, for co-culture (1# and 2#) at pH 5.5; 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl and 60 mg/L ampicillin, for co-culture (1# and 3#) at pH 7.0.

### *Preparation of Inoculum for Adaptation and Ethanol Fermentation*

In order to prepare the inoculum, a 250- mL volumetric flask containing 100 mL medium was inoculated from a fresh agar slant. The recombi-

nant *E. coli* was incubated in liquid LB medium at 37°C in a rotatory shaker at 120 rpm, and the *S. cerevisiae* 2.535 and *P. tannophilis* ATCC 2.1662 were incubated respectively in medium mentioned above at 30°C in a rotatory shaker at 80 rpm. Cells were centrifuged at 9167*g* for 10 min after growth for 24 h. The harvested cells were transferred to 100 mL medium of the same composition in 250-mL volumetric flasks. The culture was grown again for 24 h under conditions similar to those described above. Cell concentrations (dry weight) of *E. coli*, *S. cerevisiae* 2.535, and *P. tannophilis* ATCC 2.1662 were 1.9 g/L, 3.7 g/L, and 4.1 g/L respectively.

Two groups of co-cultures were utilized in the adaptation experiment and batch fermentation. One was *S. cerevisiae* 2.535 (1#) 25 mL mixed with *P. tannophilis* ATCC 2.1662 (2#) 25 mL, and the other was *S. cerevisiae* 2.535 (1#) 25 mL mixed with recombinant *E. coli* (3#) 25 mL.

### Adaptation Experiment

All adaptation experiments were conducted at 30°C, using 250-mL volumetric flasks containing 150 mL of treated softwood hydrolysate and 50 mL inoculum of co-culture. The shake flasks were sealed with 0.2 μm polyvinylidene fluoride (PVDF) film. The co-cultures were adapted for five batches. When the sugars in hydrolysate were consumed completely or the cell growth was inhibited completely, the current batch of adaptation experiment was terminated.

### Batch Fermentation Experiment

The obtained unadapted and adapted co-cultures of 1# + 2# and 1# + 3# were used in batch fermentation studies. All batch fermentations was conducted at a temperature of 30°C in 250-mL flasks, which contained 150 mL of hydrolysate and 50 mL inoculum of co-cultures. Each flask was sealed with 0.2 μm PVDF film.

### Analytical Procedures

The filtrated samples were analyzed by gas chromatography equipped with a chromosorb 105 column and a flame ionization detector for ethanol concentration. The sugar concentrations of xylose and glucose were determined by high-performance liquid chromatography (HPLC) (Knouer, Berlin, Germany) equipped with an ammonia analysis column and a refractive index detector. Acetonitrile was used as the mobile phase at a flow rate of 0.8 mL/min.

### Calculation of Fermentation Variables

The maximum theoretical yield of ethanol from sugar is 0.51 g/g. The ethanol yield (g/g) was calculated as the maximum concentration of ethanol produced divided by the concentration of sugar initially present in the hydrolysate. The volumetric productivity (g/L h) was calculated by

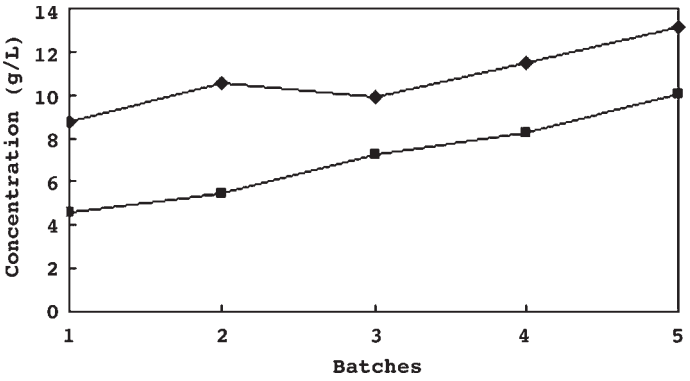


Fig. 1. The ethanol concentration of different batches during the course of culture 1# + 2# (◆) and 1# + 3# (■) adaptation.

dividing the maximum ethanol concentration by the time required to achieve such a concentration.

## Results and Discussion

### *Softwood Hydrolysate Adaptation of Microorganisms*

In each culture of *S. cerevisiae* 2.535 (1#), *P. tannophilis* ATCC 2.1662 (2#), and recombinant *E. coli* (3#), the culture *S. cerevisiae* 2.535 (1#) fails to ferment xylose to ethanol although it is able to ferment glucose to ethanol and metabolize/tolerate some inhibitors in low concentration (*13*); *P. tannophilis* (2#), which can ferment both glucose and xylose to ethanol, is sensitive to inhibitors. The recombinant *E. coli* (3#) has the ability to metabolize both glucose and xylose to ethanol. To compensate single microorganism's disadvantage, we applied the co-culture of *S. cerevisiae* 2.535 (1#), and *P. tannophilis* ATCC 2.1662 (2#), the co-culture of *S. cerevisiae* 2.535 (1#) and recombinant *E. coli* (3#) to co-ferment hydrolysate.

Figure 1 shows the results of ethanol yield during the adaptation of the microorganisms. The strains were adapted for five batches. The ethanol concentration on total sugar fermented by the adapted strains was significantly higher than that by unadapted strains. The ethanol concentration by adapted co-culture 1# + 2# and 1# + 3# increased by about 50 and 120%, respectively, after five batches in comparison with that by unadapted one. Both inhibitor tolerance and fermentation ability of microorganisms enhanced substantially, which may result from changes in gene expression and activation/inhibition of specific enzymes.

The states of adapted and unadapted strains were observed under a light microscope in a time table during the fermentation. The proportion of different microorganisms changed dramatically. After adaptation, the amount of *S. cerevisiae* 2.535 in medium was larger than other microorganisms and the cells formed clusters together with the cells of *P. tannophilis*

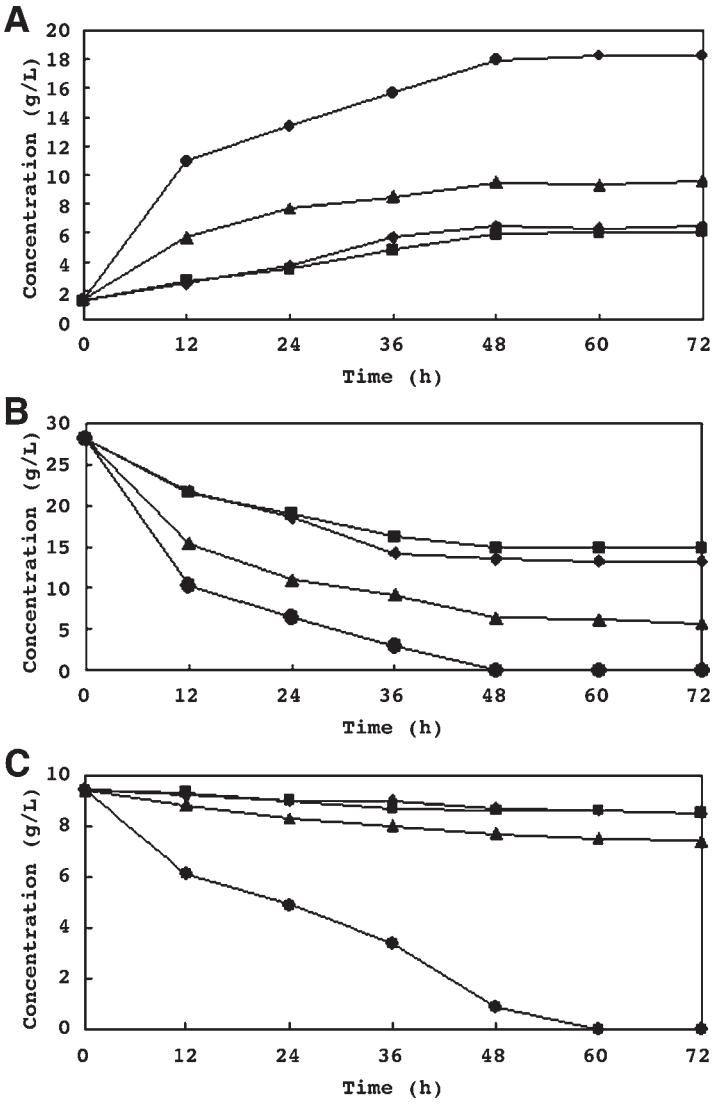


Fig. 2. Ethanol yield **(A)**, glucose consumption **(B)**, and xylose consumption **(C)** by adapted and unadapted co-culture of strains 1# and 2# using untreated and treated softwood hydrolysate. Treated softwood hydrolysate with adapted co-culture (●); Treated softwood hydrolysate with unadapted co-culture (▲); Untreated softwood hydrolysate with unadapted co-culture (◆); Untreated softwood hydrolysate with adapted co-culture (■).

ATCC 2.1662 / recombinant *E. coli* around or inside the clusters. Aggregation of *S. cerevisiae* with *P. tannophilus* or recombinant *E. coli* provides a favorable environment not only for the xylose-fermenting microorganisms, but also for more inhibitor–tolerant *S. cerevisiae*. A possible explanation is that local concentration of inhibitors decreases through inhibitor degradation by *S. cerevisiae* and increase in amount of microorganisms. During the

Table 1
Fermentation Performance of the Unadapted and Adapted Co-Culture of 1# and 2# in Untreated and Treated Softwood Hydrolysate

| | Co-culture 1# + 2# | | | |
|---|---|---|---|---|
| | Unadapted | | Adapted | |
| | Untreated | Treated | Untreated | Treated |
| Ethanol (g/L) | 6.5 | 9.6 | 6.1 | 18.2 |
| Ethanol on glucose (g/L) | 6.2 | 9 | 5.8 | 14 |
| Ethanol yield (g/g)[a] | 0.17 | 0.26 | 0.16 | 0.49 |
| Sugar consumed (%) | 42 | 65 | 38 | >99 |
| Ethanol productivity (g/L h) | 0.14 | 0.2 | 0.13 | 0.38 |

[a]Grams produced ethanol per gram total sugars

five-batch adaptation, the cell concentration (dry weight) of co-culture 1# and 2# increased from 2 g/L to 4.3 g/L, whereas that of 1# and 3# increased from 1.4 g/L to 2.7 g/L. All in all, these lead to faster sugar consumption rates and an improvement in higher ethanol concentrations.

## *Fermentation of Hydrolysate by 1# + 2#*

We tested different fermentation times and found the effective fermentation time was about 48 h (Fig. 2, Table 1). The ethanol yield on total sugar from the treated hydrolysate by adapted co-culture 1# and 2# was 0.49 g/g corresponding to 96.1% of the maximum theoretical value and the ethanol productivity was 0.38 g/L h after 48 h. The final concentration of ethanol was 18.24 g/L, possibly indicating a complete conversion of sugar mixtures to ethanol. On the contrary, ethanol yields on total sugar from the untreated hydrolysate by the unadapted and adapted co-culture 1# and 2# were only 0.17 g/g and 0.16 g/g respectively. When observed under light microscope, the growth of microorganisms was inhibited dramatically, especially for *P. tannophilis* ATCC 2.1662. Although the ethanol yield on total sugar from the treated hydrolysate by unadapted co-culture 1# and 2# was low (only 0.26 g/g) , the ethanol concentration was as high as 9.6 g/L, still much higher than untreated ones. However, 9 g/L of them came from glucose, suggesting that spreading of unadapted strains caused growth arrest of *P. tannophilis* ATCC 2.1662 cells and thereby unable to transform xylose to ethanol. These results show that the combined approach of dilute-acid softwood hydrolysate detoxification and adaptation of co-culture 1# and 2# are both significantly increase ethanol yield from the hydrolysate.

## *Fermentation of Hydrolysate by 1# + 3#*

We identified the effective fermentation time for co-culture of 1# and 3# was about 24 h (Fig. 3, Table 2). After 24 h fermentation of the treated hydrolysates by adapted co-culture 1# and 3#, the final ethanol concentration was 17.04 g/L, corresponding to an ethanol yield of 0.45 g/g, or about

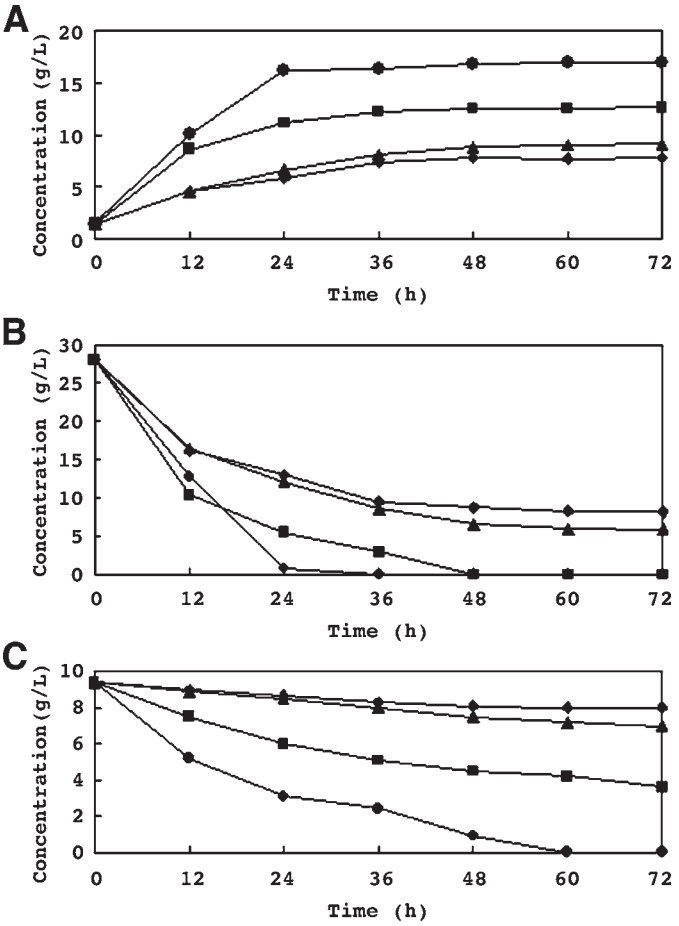


Fig. 3. Ethanol yield **(A)**, glucose consumption **(B)**, and xylose consumption **(C)** by adapted and unadapted co-culture of strains 1# and 3# using untreated and treated softwood hydrolysate. Treated softwood hydrolysate with adapted strains (●); Treated softwood hydrolysate with unadapted strains (▲); Untreated softwood hydrolysate with unadapted strains (◆); Untreated softwood hydrolysate with adapted strains (■).

88.5% of the maximum theoretical value. An ethanol productivity of 0.71 g/L h was achieved, with both glucose and xylose consumed completely. The ethanol yields on total sugar from the untreated and treated hydrolysate by the unadapted co-culture 1# and 3# were only 0.21 g/g and 0.24 g/g, and the ethanol concentrations on glucose were 7 g/L and 7.5 g/L, respectively, occupying almost all of the overall ethanol generated. Thereby glucose was the predominant consumed sugar for unadapted co-cultures. The ethanol yield on total sugar from the untreated hydrolysate by the adapted co-culture 1# and 3# was 0.34 g/g corresponding to 66% of the maximum theoretical value, the ethanol productivity was 0.53 g/L h after 24 h, and the final concentration of ethanol was 12.6 g/L, all of which are significantly higher than corresponding variables of unadapted co-cultures.

Table 2
Fermentation Performance of the Unadapted and Adapted Co-Culture of 1# and 3# in Untreated and Treated Softwood Hydrolysate

| | Co-culture 1# + 3# | | | |
|---|---|---|---|---|
| | Unadapted | | Adapted | |
| | Untreated | Treated | Untreated | Treated |
| Ethanol (g/L) | 7.8 | 9.1 | 12.6 | 17.1 |
| Ethanol on glucose (g/L) | 7 | 7.5 | 9.5 | 12 |
| Ethanol yield (g/g)[a] | 0.21 | 0.24 | 0.34 | 0.45 |
| Sugar consumed (%) | 57 | 66 | 90 | >99 |
| Ethanol productivity (g/L h) | 0.33 | 0.38 | 0.53 | 0.71 |

[a]Grams produced ethanol per gram total sugars

This supports the hypothesis that adapted co-culture of 1# and 3# has a higher tolerance to inhibitors, even though hydrolysate was not treated. Thereby, the combination of hydrolysate detoxification and microorganism's adaptation was an effective method to increase ethanol yield.

### *Comparison of Fermentation Performance of Co-Culture 1# + 2# and 1# + 3#*

The goal of the experiment was to select a co-culture that is able to metabolize both glucose and xylose to ethanol and able to minimize the negative effects of inhibitors in the softwood dilute-acid hydrolysate on fermentation. Combination of strain traits could make the best use of sugar in softwood dilute-acid hydrolysate to ethanol. Adaptation of *S. cerevisiae* 2.535, combined with *P. tannophilis* ATCC 2.1662 or recombinant *E. coli* on softwood dilute-acid hydrolysate is an efficient way to increase ethanol yield. The adapted co-cultures of 1# + 2# and 1# + 3# with hydrolysate both increase in both sugar consumption and ethanol yield (shown in Fig. 2, Table 1, Fig. 3, and Table 2). The effective fermentation time for the co-culture 1# + 2# and 1# + 3# was 48 h and 24 h with ethanol productivity 0.38 g/L h and 0.71 g/L h respectively. The adapted co-culture 1# + 3# reached better results than adapted co-culture 1# + 2# under the condition that hydrolysate is not treated. This might be that the recombinant *E. coli* has a higher tolerance to inhibitors and more efficiency of ethanol productivity than that *P. tannophilis* ATCC 2.1662 has. It might be possible that more inhibitors might be removed in pH 7.0 condition of untreated hydrolysate than in pH 5.5. However, the ethanol yield from the treated hydrolysate by adapted co-culture 1# + 2# was higher than adapted co-culture 1# + 3# with treated hydrolysate, possibly suggesting that the recombinant *E. coli* partially converted sugar to produce the byproduct.

## Conclusion

Two groups of co-culture, *S. cerevisiae* 2.535 (1#) and *P. tannophilis* ATCC 2.1662 (2#), *S. cerevisiae* 2.535 (1#) and recombinant *E. coli* (3#), were adapted for five batches. After adaptation, the ethanol concentrations by the adapted co-culture 1# + 2# and 1# + 3# increased by about 50 and 120%, respectively, in comparison the unadapted. Both inhibitor tolerance and fermentation ability of microorganisms enhanced substantially, which may result from changes in gene expression and activation/inhibition of specific enzymes. Observation under light microscope shows that the amount of *S. cerevisiae* 2.535 in medium was larger than that of other microorganisms, and that cells formed clusters together with cells of *P. tannophilis* ATCC 2.1662/recombinant *E. coli.* Aggregation of *S. cerevisiae* with *P. tannophilus* or recombinant *E. coli* provides a favorable environment not only for the xylose-fermenting microorganisms, but also for the more inhibitor tolerant *S. cerevisiae*, which may be as a result of the local inhibitor concentration of inhibitors was reduced through inhibitor degradation by *S. cerevisiae*. This favorable environment may lead to an improvement in overall sugar conversion and final ethanol concentrations.

The dilute-acid softwood hydrolysate, detoxified and then supplemented with mineral salts and nutrients, was subsequently further fermented to ethanol by both the adapted co-culture of *S. cerevisiae* 2.535 (1#) and *P. tannophilis* ATCC 2.1662 (2#), and that of *S. cerevisiae* 2.535 (1#) and recombinant *E.coli* (3#). On one hand, batch fermentation of softwood hydrolysate helped to generate maximal ethanol yields and optimize ethanol productivities for both adapted co-culture 1# and 2# and adapted co-culture 1# and 3#. On the other hand, our results shown in Table 1 and 2 also demonstrated that combined detoxification procedure at least partially counteracts the negative effects of inhibiting substances present in hydrolysate on fermentation. Therefore, the combination of hydrolysate detoxification and metabolism adaptation should be an effective method to optimize ethanol yields.

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