2011 2nd International Conference on Biotechnology and Food Science
IPCBEE vol.7 (2011) © (2011) IACSIT Press, Singapore

Impact of Vanilin in Hydrolysate of Corn Stalk on L-Lactic Acid Fermentation

Zhe Liu, Ruifeng Yang,Jie Lu*, Hui Jin
Dalian Polytechnic University
Dalian, China
lujie@dlpu.edu.cn

***Abstract*-During the liquid hot water pretreatment of lignocellulosic materials such as corn stalk, hemicellulose is hydrolyzed into monosaccharides, and meanwhile, toxic by-products are simultaneously generated, which may influence L-lactic acid fermentation. Studies on the inhibitory effects of the by-products on L-lactic acid fermentation are of practical use for further improvement of L-lactic acid yield from lignocellulosic materials. In this study, one by-product, vanillin, was identified in the hydrolysate of local corn stalk, which was added into the medium at different concentrations to study their impacts on the growth and L-lactic acid fermentation of a *Rhizopus oryzae* (AS3.819). The work indicated that L-lactic acid production was inhibited by vanillin to a lesser extent than that to the growth. Vanillin caused a much longer lag-phase in growth when the concentration was 0.8 g/l, which showed remarkably inhibition effect, and the lag-phase was not obvious at lower concentrations. At the concentration of 1 g/l, vanillin completely inhibited the fermentation as well as the cell growth.**

Keywords-corn stalk; vanillin;hydrolysis; by-products; L(+)-lactic acid

I. INTRODUCTION

For the past several years, the producers of raw agricultural commodities have suffered from low prices and surpluses because of reduced demand and increased world supplies. Recently efforts have been made to reverse the trend by converting agricultural commodities into value-added products. For example, corn was grown in the 1970's mainly for livestock feeds, but today it is being used increasingly to produce high-fructose corn syrup, fuel-alcohol and other non-food products .

Lactic acid is produced commercially by the fermentation of glucose, molasses or cheese whey with homofermentative lactic acid bacteria. Lockwood reported that selected strains of *Rhizopus oryzae* in surface culture converted D-glucose in a chemically defined medium to a large amount of L(+)-lactic acid in the presence of calcium carbonate. Prescott and Dunn have reviewed the production of L(+)-lactic acid by molds.

Lignocellulosic material from agricultural waste such as corn stalk represents an abundant renewable energy source for bioconversion processes as well as a raw material for the paper and pulp industry. [1]

Several studies have been reported on the use of pure glucose solutions as fermentation substrate for obtaining L-lactic acid, although more profitable processes are based on the hydrolysis of the cellulosic fraction of some lignocellulosics, which can be selectively converted into glucose solutions by a mild acid hydrolysis to obtain culture media useful for bioconversion into L-lactic acid.[2] Before corn stalk can be utilized, a pretreatment is necessary to break down the lignocellulose into the major polymeric constituents: cellulose, hemicellulose and lignin. Numerous pretreatment methods have been proposed and investigated, e.g. alkaline treatment, steam treatment with or without SO_2, ammonia fiber explosion, ammonia-recycled percolation, lime pretreatment or dilute acid pretreatment [3]. One of the most promising pretreatment processes for lignocellulosic materials (LCM) is liquid hot water (LHW) [4] that has been gaining increasing attention as both an environmentally friendly solvent and attractive reaction media for a variety of applications.

Depending on the process conditions, most of the hemicellulose and lignin degradation products will be dissolved and the cellulose will be recovered in the solid fraction. The celllulose and hemicellulose both can be hydrolyzed and used for fermentation purposes, but the usage of the hemicellulose fraction is more complicated due to the fact that xylose, which is the dominating monosaccharide released, is difficult to ferment.[5] At the same time, it is possible to form some by-products during the relatively harsh pretreatment. [6]

Rhizopus oryzae is a lactic acid producing filamentous fungus. This fungus can produce lactic acid, fumaric acid, and ethanol depending on the cultivation condition [7–9].

In this study, the hydrolysate from corn stalk treated with LHW was used as starting material for production of L-lactic acid by *Rhizopus oryzae*. The components of the hydrolysate were analyzed by HPLC, and the content of vanillin was related higher, about 0.4~2.0 g/l. So the influence of vanillin at different concentrations on L-lactic acid fermentation and the growth of *Rhizopus oryzae* were studied to provide basis and references for the L-lactic acid fermentation from the hydrolysate.

So the influence of vanillin at different concentrations on L-lactic acid fermentation and the growth of *Rhizopus oryzae* were studied to provide basis and references for the L-lactic acid fermentation from the hydrolysis.

II. EXPERIMENTAL

A. Microorganism

Rhizopus oryzae (AS3.819), purchased from Institute of Microbiology (Beijing, China). The Rhizopus *oryzae* was maintained on PDA solid medium as described in L-lactic acid production by pellet-form *Rhizopus oryzae* in a stirred tank fermentor[10] supplemented with 20 g/l glucose. .

B. Fermentation media

The strain was grown on Erlenmeyer flasks containing 100 ml of the complete media. (glucose 80 g/l, $(NH_4)_2SO_4$ 2 g/l, KH_2PO_4 0.3 g/l, $ZnSO_4 \cdot 7H_2O$ 0.05 g/l, $MgSO_4$ 0.3 g/l, $CaCO_3$ 45 g/l , sterilized separately, natural pH.)

C. Substrates

LHW was prepared as previously described [8]. In all experiments, the hydrolysate was supplemented with all other components of the fermentation media except for the primary carbon source.

LHW was carried out by addition of water to the whole corn stalk powder (40-60 mesh) and applying 190℃ for 20min in a closed container. After cooling to room temperature, the pH was adjusted to 6.5 by addition of 10 M NaOH.

To investigate the impact of single factor on the L(+)-lactic acid fermentation, the simulation medium was made, by adding the inhibitor at different concentration to the fermentation medium.

D. L-Lactic acid fermentation

Hydrolyzates selected to ferment were neutralized with $CaCO_3$ to a final pH of 6.5. Five milliliters of inoculums were added to 100 ml of culture media to carry out the fermentations assays. Experiments were performed as batch fermentations under aerobic conditions with 50 ml liquid medium in 250 ml flask, using 10% (v/v) inoculums. Added the vanillin of different concentrations to the cultivation medium, *Rhizopus oryzae* was inoculated at their optimum temperature 34 ℃ for 72 h.

All cultivations were carried out as triplicate experiments and all variations were less that 10%.

E. Analytical techniques

Samples taken during fermentation were centrifuged using an IEC Micromax centrifuge at 20,000 rpm for 10 min to remove solids. Sugars and L-lactic acid were detected on a SBA-40D Bio-sensing Analyzer (Biology Institute of Shandong Academy of Sciences)

The biomass is determined by the bacteria dry quality method [9]. The residue gained from the fermentation liquid was washed by 0.01 M diluted hydrochloric acid, removed the overweight $CaCO_3$, washed with distilled water until the pH is neutral, then it was measured by oven-drying to constant weight at 80℃.

III. RESULTS AND DISCUSSION

A. Effect of vanillin on glucose consumption

During the process of L-lactic acid fermentation, the enzyme inverted as well as *Rhizopus oryzae* growthing. It produced energy and other nutrients for itself using.

Figure 1(a) showed the influence of fermentation time on the glucose concentration during the fermentation process of different vanillin concentrations. Figure 1(b) showed the vanillin concentration on the glucose concentration at different time. It can be seen from the Figure 1(a) that the influences of vanillin were not consistent at different phase of fermentation. Before 24h, at the beginning of fermentation, the glucose consumption was slow. Between 24 to 72 h, the glucose content sharply reduced with time, until it was consumed completely. Besides that, the addition of vanillin raised the rate of glucose consumption before 24 h. At the later stage, the glucose consumption was faster when the vanillin concentration was lower than 0.8 g/l. But when the vanillin concentration was higher than 0.8 g/l, the glucose consumption gradually slowed down as the vanillin concentration increasing. When the concentration of vanillin is low, the glucose can relatively be consumed completely. At vanillin concentration of 0.8 g/l, the inhibitory action is quite obvious. It can be seen from the figure 1(b) that the vanillin may lead to better consumption of glucose at the same time when its concentration was lower than 0.8g/l.

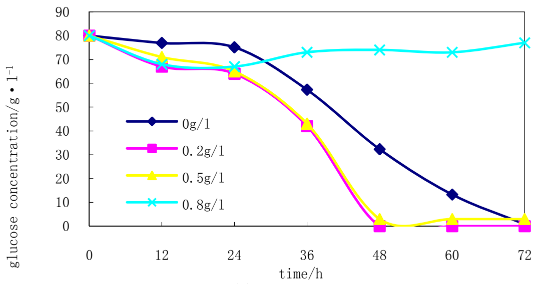
It can be found that, after 72 h, when the concentration of vanillin was 0, 0.2 and 0.5 g/l, the glucose can be consumed completely. And when it was 0.8 g/l and 1g/l, there were not evident change on the concentrations of glucose.

In the process, the consumption of glucose was mainly conducted by *Rhizopus oryzae*. *Rhizopus oryzae* need some time to adapt the environments of culture medium. During the period, it presented some special characteristic, such as slow growth and inactive reproductive capacity. Once *Rhizopus oryzae* was accommodated to circumstances, it breeds quickly, consuming a mount of glucose for itself assimilation. For the adding of vanillin, the cell growth and multiplication were inhibited. So *Rhizopus oryzae* inoculated in the culture medium took longer time to adapt new circumstance, and the glucose consumption is little. High vanillin concentration result in the low survival rates of *Rhizopus oryzae*, which is adverse to the glucose degradation.

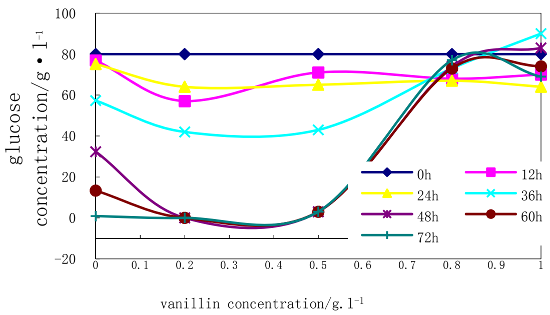
B. Effect of vanillin on L-lactic acid production

The production of L-lactic acid by *Rhizopus oryzae* at the different vanillin concentrations was shown in Figure 2. After 72 h of fermentation, the concentration of L-lactic acid was relatively dependent on the initial vanillin concentration, with a decrease form 45 g/l to 14 g/l.

Figure 2 showed that the impacts of fermentation time on the L-lactic acid production during the fermentation process of different vanillin concentrations were different. In the fermentation liquid without vanillin, the L-lactic acid content increasing rapidly after 24 h, and basically reached the maximum at 60 h, which is 45.2 g/l. When 0.2 g/l vanillin was presented in the culture medium, the L-lactic acid content increasing rapidly after 36 h, and basically reached the maximum at 48 h, which is 29 g/l. When 0.5 g/l vanillin was presented, the L-lactic acid content increasing rapidly after 24 h, and basically reached the maximum at 48h, which is 31 g/l. When 3 g/l vanillin was presented, the L-lactic acid content increasing rapidly after 60 h, and basically reached the maximum at 72 h, which is 13.8 g/l. However, when vanillin is 0.8 g/l and 1g/l, the L-lactic acid content were about 14 g/l and no obvious change during the 72 h.



(a)



(b)

Figure1. Effect of vanillin on glucose consumption

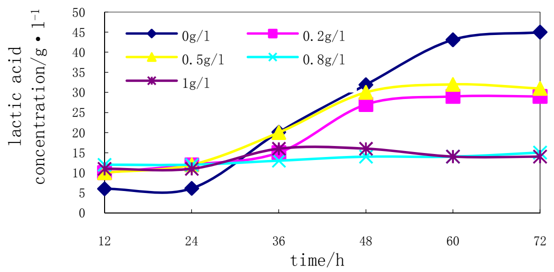


Figure 2 Effect of vanillin on L-lactic acid concentration

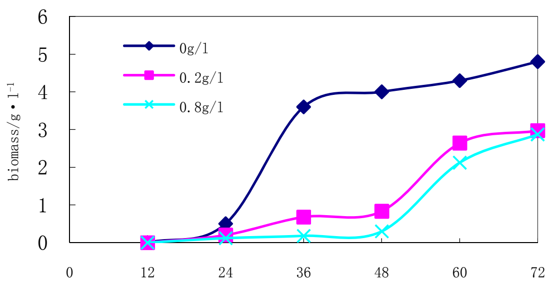
The existence of vanillin can inhibit the process of L-lactic acid production. It can both reduce the yield of L-lactic acid and extend the start time of the L-lactic acid generation; the more concentration of vanillin, the lower generation of L-lactic acid and conversion rate of glucose.

C. Effect of vanillin on cell growth

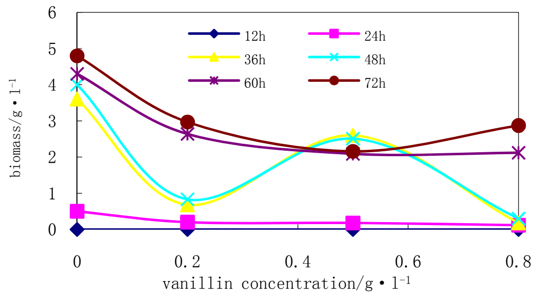
The biomass gained in the process of *Rhizopus oryzae* growthing. It was related to the production of L-lactic acid.

Figure 3(a) showed the biomass changed with the time, and Figure 3(b) showed the biomass changed with the concentration of vanillin.

As shown in Figure 3, the cell growth rates were gradually reduced with the increasing of vanillin concentration, which have great influence on the biomass. In the broth without vanillin, the biomass rapidly increased after 24 h, became slowly at 48 h, and reached 4.8 g/l at 72h. 0.2 g/l vanillin made the lag phase of cell growth obviously extended to 48 h, and reached 3 g/l at 72 h. When the concentration of vanillin was 0.8 g/l, the biomass rapidly increased after 48 h and reached 3 g/l at 72 h.



(a)



(b)

Figure 3. Effect of vanillin on biomass

D. Effect of vanillin on the fermentation of Rhizopus oryzae

From table 1, it was observed that L-lactic acid yield decreased as the vanillin concentration increasing, but it did change a lot at different vanillin. And the glucose conversion rate rose when the vanillin concentration is 0.5 and 0.8g/l. L-lactic acid yield of 42.5% and 18.75%, and complete substrate utilization were achieved with the vanillin concentration of 0.2 g/l. This suggests that the existence of vanillin can increased the consumption of glucose, but not for the production of L-lactic acid, so the reason of glucose consumption and the resultant of this reaction need a further study.

IV. CONCLUSION

There was significant inhibitory effect of the vanillin on production of L-lactic acid from glucose. It can both reduce the yield of L-lactic acid and extend the start time of the L-lactic acid generation; the more concentration of vanillin, the lower generation of L-lactic acid and conversion rate of glucose. The vanillin concentrations have impact on the biomass of *Rhizopus oryzae*. The larger concentration of vanillin made the lag phase of cell growth obviously extended and the yield of biomass dropped.

TABLE I. EFFECT OF VANILLIN ON THE FERMENTATION OF *RHIZOPUS ORYZAE*

Vanillin concentration /g.l^{-1}	glucose conversion rate /%	L-lactic acid yield (YL/CS) /%
0	99	56.25
0.2	100	36.25
0.5	96.25	42.5
0.8	3.75	18.75
1	13.75	17.5

Note: YL/CS (L-lactic acid yield) = [L-lactic acid]max / [sugar]consumed

ACKNOWLEDGMENT

Contract grant sponsor: science research plan of the Educational Department of Liaoning Province（2008S019） and Dalian Science and Technology Fund (2007J23JH019).

REFERENCES

[1] Arivid G, Gunnar J. Lactic acid production from wheat straw hemicellulose hydrolysate by Lactobacillus pentosus and Lactobacillus brevis. Bioresource Technology, 2002, 81, 217-223.

[2] Bustos G, de la Torre N, Moldes AB, Cruz JM, Domı´nguez JM. Revalorization of hemicellulosic trimming vine shoots hydrolyzates trough continuous production of lactic acid and biosurfactants by L. pentosus. J Food Eng , 2007, 78:405–412.

[3] C.S. Gong, N.J. Cao, J. Du, G.T. Tsao, Ethanol production from renewable resources, Adv. Biochem. Eng. Biotechnol. 65, 1999, 207 −241.K. Elissa, “Title of paper if known,” unpublished.

[4] L.R. Lynd, Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment, and policy, Annu.Rev. EnergyEnviron. 21 , 1996, 403−465.

[5] Lee, J..Biological conversion of lignocellulosic biomass to ethanol. J. Biotechnol, 1997, 56,1-24

[6] Olsson, L., Hahn-Hagerdal, B., Fermentation of lighocellulosic hydrolysates for ethanol production. Enzyme Microb. Technol, 1996, 18, 312-331.

[7] Carta FS, Soccol CR, Ramos LP, Fontana JD (1999) Production of fumaric acid by fermentation of enzymatic hydrolysates derived from cassava bagasse. Bioresour Technol 68:23–28

[8] Christen P, Bramorski A, Revah S, Soccol CR (2000) Characterization of volatile compounds produced by Rhizopus strains grown on agro-industrial solid wastes. Bioresour Technol 71:211–215

[9] Kitpreechavanich V, Maneeboon T, Kayano Y, Sakai K Comparative characterization of L-lactic acid-producing thermotolerant Rhizopus fungi. J Biosci Bioeng, 2008, 106:541–548

[10] Dong Mei Bai, MinZe Jia. L(+)-lactic acid production by pellet-form Rhizopus oryzae R1021 in a stirred tank fermentor [J]. Chemical Engineering Science, 2003,58: 785-791