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Optimization of lactic acid fermentation from distillers grains hydrolysates using orthogonal experimental design (OED)

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ABSTRACT

Lignocellulosic biomass-derived sugars is considered to be an economically attractive carbohydrate feedstock for large-scale fermentation of bulk chemicals such as lactic acid (LA). The aim of this study was to investigate the possibility of LA production from distillers grains hydrolysates (DGHs) by the Rhizopus oryzae and to optimize the biological conversion of reducing sugar into LA to evaluate the culture conditions. The effects of factors such as inoculations size, $CaCO_3$ addition, pH value and fermentation time on the lactic acid concentration (LAC) and the reducing sugars utilization rate (RSUR) were researched by the method of orthogonal experimental design (OED). The results show that the optimal fermentation parameters are inoculation size of 3.0% seed culture, $CaCO_3$ addition of 80 $g \cdot L^{-1}$, fermentation time of 96 h and culture pH of 6.5. This study provides an encouraging means of producing LA from the low-cost waste distillers grains.

Keywords: *Rhizopus oryzae*, Distiller grains hydrolysates (DGHs), Lactic acid (LA), Reducing sugars, orthogonal experimental design (OED)

INTRODUCTION

Lactic acid (LA) is the most widely occurring hydroxycarboxylic acid, having a prime position due to its versatile applications in food, pharmaceutical, textile, leather, and chemical industries. Highly purified, preferably LA anhydrous monomer is required for the production of the biodegradable polymer polylactic acid (PLA), which is an environmentally friendly replacement of plastics derived from petrochemical materials. As the physical properties of PLA depend on the isomeric composition of LA, the production of optically pure LA is essential for polymerization[1]. LA can be produced by fossil oil-derived chemical synthesis or microbial fermentation processes. In contrast with chemical synthesis[2,3], the biotechnological production offers several advantages like low cost of substrates, low production temperature, and low energy consumption. Moreover, high product specificity is yet another advantage of LA fermentation, as it produces a desired stereoisomer, optically pure L-(+)- or D-(−)-lactic acid.

Many microorganisms have proven ability to produce LA, including filamentous fungi, bacterial species and various gene modified strains[4-6]. Lactic acid bacteria require a strict nutrient environment and growth medium with complex supplements, such as amino acids, vitamins and other growth factors, and the product often becomes the racemic mixture of LA, which adds to the costs of LA production and complicates purification of LA. In contrast, *Rhizopus* species has many advantages over the bacterial process such as the use of a chemically defined medium and waste materials, simplifing product purification, the ability to metabolize high concentrations of glucose, little pH maintenance required, no need of specific nutrients and also produce enantiomerically pure LA. Especially *Rhizopus oryzae* fungus, an obligate aerobe, have recently been the most preferred microorganism for LA fermentation.

The economics of production of LA is dependent on many factors of which the cost of raw material is very significant. To enhance the productivity and economy of LA production, many extensive studies have investigated the potential of utilizing less costly raw materials, such as starchy, cellulosic materials and molasses. Among the above-mentioned raw materials, lignocellulosic biomass is an inexpensive and widely available renewable carbon source on account of large amounts of fermentable sugars.To date, cassava pulp, wheat straw, paper waste, corn stover, corn cob, wood, sugar beet molasses, have all been used as a carbon source for *R. oryzae* in the production of LA[7-9].

Distillers grains, one of the lignocellulosic biomasses, is an available byproduct in the Chinese liquor industry. Distillers grains are rich in cellulose and hemicellulose that has been used as a cheap source of raw material for production of value-added products by saccharification and microbial fermentation processes, such as xylitol, bioenergy, etc[10]. Despite of rich nutritional composition, distillers grains hydrolysates (DGHs) by saccharification has not been tested as a fermentation substrate for production of organic acid.

Therefore, in this regard, the present study was undertaken to investigate the feasibility and ability of using DGHs as carbon source to produce LA and statistically study the optimal fermentation condition with respects of inoculation size, $CaCO_3$ addition, growth pH, and fermentation time by the method of orthogonal experimental design (OED).

EXPERIMENTAL SECTION

Raw material

Distillers grains was kindly supplied by Jinhui Liquor Co.Ltd and dried at room temperature to equilibrium moisture content. The chemical compositions were as follows: cellulose 32.08%, lignin 11.23%，hemicelluloses 12.64%.

Microorganism and Culture Media

The microorganism used in this study, *Rhizopus oryzae* CICC4113, was purchased from the China Center of Industrial Culture Collection (CICC).

The composition of the pre-culture medium was as follows ($g \cdot L^{-1}$): glucose, 50; NH_4NO_3, 2; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.25; $ZnSO_4 \cdot 7H_2O$, 0.05 and $CaCO_3$, 10. The composition of the fermentation medium was 100 mL supernatant of DGHs with ($g \cdot L^{-1}$): KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5 and $ZnSO_4 \cdot 7H_2O$, 0.04. All culture media were autoclaved at 121°C for 20 min.

Culture Method

For fermentation study, the sporangiospores were collected from the 7-day culture on a PDA plate by shaving and extracting the spores with sterile water. The spore concentration was determined by spore counting using a haemacytometer before inoculation. The stock spore suspension was stored at -80 °C. A 250 mL flask containing 100 mL of the pre-culture medium was inoculated with a final concentration of 3×10^6 spores/mL and incubated in a rotary shaker (200 $r \cdot min^{-1}$) at 30°C for 12 h. This overnight culture, as seed culture, was used to inoculate the fermentation medium.

Preparation of distillers grains hydrolysates (DGHs)

The DGHs used for fermentation was obtained by acid hydrolysis using mixed acid as catalysts. The distillers grains feedstock was primarily mixed and impregnated with mixed acid solution at solid-liquid ratio of 1:12 ($g \cdot mL^{-1}$). The mixed acid was composed of hydrochloric acid and phosphoric acid with equal mass proportion. The mixtures were allowed to stand for 12 h at 25°C in order to equilibrate the mixed acid concentration between the bulk phase and feedstock. Acid hydrolysis was performed in the flasks, which were placed inside the autoclave. The parameters selected for the hydrolysis experiments were mixed acid concentration (2.0%,w/w), hydrolysis time (120 min) and hydrolysis temperature (100°C). After hydrolysis was completed, the acidic hydrolysis residues were separated from the hydrolysates by 10 min of centrifugation at 3000 r/min. The liquid phase was used as the substrate for fermentation.

Fermentation of LA from DGHs

Unless otherwise noted, all fermentations experiment were carried out in 250-mL Erlenmeyer flasks containing 50 mL fermentation media operated at 30°C on a rotary shaker at 200 r/min. The effects of nitrogen source, inoculation size, $CaCO_3$ addition, growth pH, and fermentation time on the lactic acid concentration (LAC) and the reducing sugars utilization rate (RSUR) was researched. The culture broth was centrifuged at 12000 $r \cdot min^{-1}$ for 10 min and the resulting supernatant was used for measurement of the surplus reducing sugars and LA. LAC was determined using p-Hydroxybiphenol Colorimetry. The reducing sugars were described as the sum of the total sugars content and determined by the dinitrosalicylic acid method using glucose as the standard. The LA yield and the RSUR were

calculated using Eq.1 and Eq.2.

$$\text{The LA yield (\% by weight)} = \frac{\text{grams of lactic acid produced}}{\text{grams of reducing sugars consumed}} \times 100\% \quad (1)$$

$$\text{Reducing sugars utilization rate (\% by weight)} = \frac{\text{grams of reducing sugars consumed}}{\text{grams of total reducing sugars}} \times 100\% \quad (2)$$

Statistical analysis

All of the experiments were performed in triplicate. The data are expressed as mean ± standard deviation (SD) and the significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

Effects of pH on LA production

Medium pH is accepted as one of the most important operational factors affecting LA production from *Rhizopus* species. In order to determine the impact of growth pH on the fermentation of LA, the growth pH was controlled at 4.5, 5.0, 5.5, 6.0 and 6.5. The results presented in Fig.3 depicts the effects of pH on the LAC and the RSUR. Both the amount of LA and the RSUR increased gradually along with a increasing pH value when pH was blow 6.0, while approximately 10.82 $g \cdot L^{-1}$ of LA was the maximum concentration and the LA yield based on the reducing sugars was 0.49 $g \cdot g^{-1}$ in the fermentation systems. The results indicated that the higher the pH value, the more LA could be produced by *Rhizopus oryzae*, which could be due to the promotion of forming lactate at high pH that could break the reaction equilibrium. On the other hand, pH could affect the morphology of *Rhizopus oryzae* in fermentation, which could consequently affect the production of LA. High pH could make *Rhizopus oryzae* produce spherical pellets and achieve high LA yield while low pH could agglomerate the biomass and generate filamentous mycelia with low LA yield[11].The results obtained from the current research were identified with those of previous studies. According to Miura et al., the optimal pH range was 6~6.5 for the highest yield of LA production by *Rhizopus sp*[12]. Huang et al. recognized that the best pH for *Rhizopus* fungi fermentation would be in the range of 5.0~6.0[13].

Effects of inoculum size on LA production.

To study the effects of inoculum size on LA production, *Rhizopus oryzae* was inoculated with different volumes of seed culture, from 1.0% to 3.0%. The effects of inoculum size on the LAC and the RSUR are presented in Fig. 4. As showed in Fig.4, the LAC increased from 4.07 $g \cdot L^{-1}$ to 9.06 $g \cdot L^{-1}$ with the increase of inoculum size from 1.0% to 2.5%. If continue to increase inoculum size, a sharp decrease in both the LAC and RSUR were observed when the inoculum size was higher than 2.5%, due to more than 2.5% spore suspension led to flocculation of fungi mycelia. The LAC was highest (9.06 $g \cdot L^{-1}$) with the highest RSUR (62.89%) when the inoculum size of spore suspension was 2.5%. It could be indicated that the highest RSUR resulted in the highest LA yield (0.41$g \cdot g^{-1}$), due to more reducing sugars was transformed for fungi metabolization. This conclusion agrees well with Wu et al.[14]. From these studies, the optimum inoculum size was 2.5.

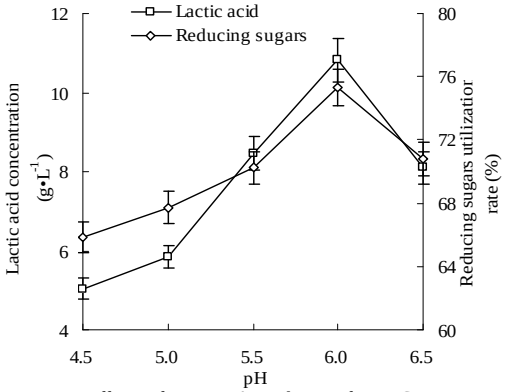

Fig.1 Effects of pH on LA production from DGHs by *R. oryzae*

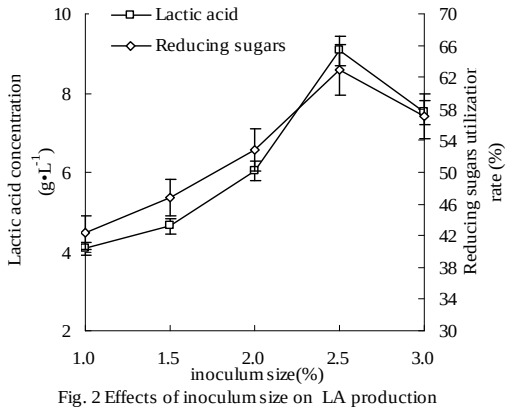

Fig. 2 Effects of inoculum size on LA production from DGHs by *R. oryzae*

Effect of fermentation time on LA production

The observation of *Rhizopus oryzae* in consuming reducing sugars and producing LA is presented in Fig. 5. There were three fermentation phases, lag, exponential and declining phases in terms of LA synthesis during the course of

96 hours cultivation. During the 12 st hour of growth, the fungus was adjusting itself to the new incubation environment without showing substantial changes in concentrations of either reducing sugars or LA in the culture medium. In the next 84 hours, the LAC was linearly increasing, indicated by the major uptake of reducing sugars during this period. The fungus started to accumulate biomass and achieved a maximal LAC (10.85 $g \cdot L^{-1}$) with the RSUR of 75.86%, exponential phase for LA synthesis showed the similar trend with the RSUR. By comparison of the production results in 12 st hour and 96 st hour fermentation presented in Fig. 5, the LA yield increased from 0.24 $g \cdot g^{-1}$ to 0.51 $g \cdot g^{-1}$ using *Rhizopus oryzae*. In the following 12 hours, there was almost no additional LA produced (the level stayed at around 10.59 $g \cdot L^{-1}$ all the way through), indicating that prolonged incubation times did not increase the LAC. In brief, the results exhibited that the highest LAC were reached within 96 hours and the decreasing of LAC were observed after 96 th hour. In general, 96 hours of incubation were sufficient for the system to reach the stage of highest yield of LA.

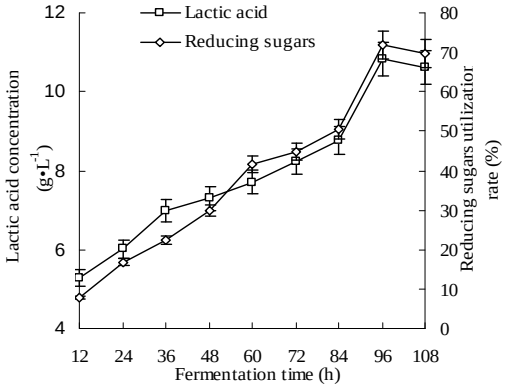

Fig.3 Effects of fermentation time on LA production from DGHs by *R. oryzae*

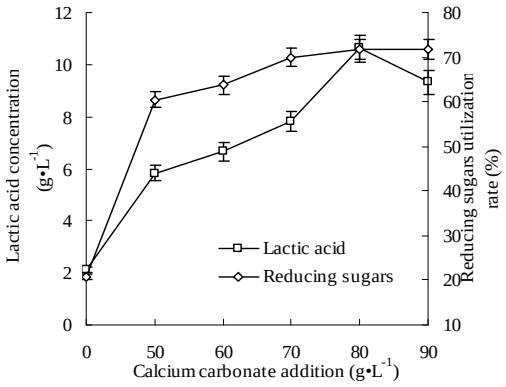

Fig.4 Effects of calcium carbonate addition on LA production from DGHs by *R. oryzae*

Effect of $CaCO_3$ addition on LA production

Lactic acid is known to be a strong inhibitor for both cell growth and LA production. Calcium carbonate ($CaCO_3$) is a commonly used reagent to neutralize lactic acid during fermentation. Its low solubility in water makes it possible to neutralize LA and maintain the pH at certain level automatically[14-15].

The LAC and the RSUR for different $CaCO_3$ addition are presented in Fig 6. In this study, a control experiment with no $CaCO_3$ addition was carried out by setting up an initial pH at 6.0 and the growth pH during the fermentation was not adjusted. As indicated in Fig. 6, compared with no $CaCO_3$ addition, the addition of $CaCO_3$ enhanced significantly the microbial performances of the metabolic fermentation of LA. The reasons could be that the alkali neutralizing LA can easily move fermentation toward LA metabolism. Moreover, an obvious increase in the LAC and the RSUR were found with the $CaCO_3$ dosage, increasing from 5.82 $g \cdot L^{-1}$ to 10.63 $g \cdot L^{-1}$ and from 60.47% to 71.86%, respectively. However, a dosage of more than 80 $g \cdot L^{-1}$ $CaCO_3$ resulted in a slight decrease in LA yield, from 0.48 $g \cdot g^{-1}$ to 0.42 $g \cdot g^{-1}$. Therefore, the supplementation of 80 $g \cdot L^{-1}$ of $CaCO_3$ may be sufficient for maintaining a growth pH to achieve an optimum LA production.

Optimation of fermentation condition of LA production

In order to obtain higher LA productivity needed in fermentation by *R. oryzae*, the fermentation conditions were optimized through the $L_9(3)^4$ orthogonal experimental design, and each fermentation was tested in triplicate. Four factors, namely inoculation size, $CaCO_3$ addition, growth pH, and fermentation time in three levels, were evaluated and optimized (Table.1). K_1~K_3 and k_1~k_3 were the LAC and the RSUR respectively under the various investigated conditions, and the maximum value was the optimum value. In addtition, according to the largest donating rule, the factor with the largest range value (K_{max}-K_{min} and k_{max}-k_{min}) have the greatest effect on the LA fermentation. Table.1 shows that the rank order of the four influential factors are D>C>A>B for LA C and C>A>D>B for the RSUR, respectively.

To evaluate which factors had the greatest influence on LAC and the RSUR, variance analyses were applied to assess the results. The result Table.2 and Table.3 indicated that fermentation time (factor D) and inoculation size (factor C) had the greatest impact on the LAC and the RSUR, respectively. The pH value (factor D) had the least influence for both indexes. The variance analyses result was in good agreement with what was observed in Table.1. Considering overall, the optimum fermentation conditions were $A_2B_3C_3D_2$ for both indexes, that is the maximal LAC and the RSUR were obtained when the $CaCO_3$ addition was 80 $g \cdot L^{-1}$; pH value was 6.5; the inoculation size was 3.0% (10^8 spores/mL); and the fermentation time was 96 h.

Table.1 $L_9(3)^4$ Orthogonal design and experimental results

	A: $CaCO_3$ addition ($g \cdot L^{-1}$)	B: pH	C: Inoculation size (%)	D: Fermentation time (h)	LA C ($g \cdot L^{-1}$)	RSUR (%)
1	1(70)	1(5.5)	1(2.0)	1(84)	7.24	64.82
2	1	2(6.0)	2(2.5)	2(96)	8.96	70.09
3	1	3(6.5)	3(3.0)	3(108)	9.29	80.77
4	2(80)	1	2	3	8.62	70.88
5	2	2	3	1	9.25	76.37
6	2	3	1	2	12.15	69.25
7	3(90)	1	3	2	12.79	77.37
8	3	2	1	3	8.07	58.32
9	3	3	2	1	7.96	64.48
K_1	8.5	9.6	9.2	8.2	the rank order of influential factors for	
K_2	9.9	8.8	8.5	11.3	the LAC: D> C> A > B	
K_3	9.6	9.8	10.4	8.7	the optimum fermentation conditions:	
R	1.4	1.0	1.9	3.1	$A_2B_3C_3D_2$	
k_1	71.9	71.0	64.1	68.6	the rank order of influential factors for the RSUR: C> A >	
k_2	72.2	68.3	68.5	72.2	D> B	
k_3	66.7	71.5	78.2	70.0	the optimum fermentation conditions:	
r	5.5	3.2	14.1	3.6	$A_2B_3C_3D_2$	

Table.2 Variance analyses statistical data on the factors of LA yield

source	Sum of square	Degree freedom	Mean square	F	Significance [a]
$CaCO_3$ addition	3.672	2	1.836	0.214	0.824
pH	1.768	2	0.884	0.103	0.907
Inoculation size	5.799	2	2.899	0.338	0.747
Fermentation time	17.152	2	8.576	2.985	0.253
Total	818.563	9			
Corrected	28.391	8			

a: P-value is less than 0.05 being regarded as significant.

Table.3 Variance analyses statistical data on the factors of RSUR

source	Sum of square	Degree freedom	Mean square	F	Significance
$CaCO_3$ addition	56.433	2	28.217	3.074	0.245
pH	18.361	2	9.180	0.889	0.529
Inoculation size	309.905	2	154.952	16.879	0.056
Fermentation time	20.644	2	10.322	1.124	0.471
Total	44834.957	9			
Corrected	405.343	8			

a: P-value is less than 0.05 being regarded as significant.

CONCLUSION

As the increasing interest in producing biotechnological products from low-cost and renewable biomass, the production of LA from lignocellulosic biomasses has gained considerable interest recently. Distillers grains, which is a waste and low-cost by-product in the liquor production, contains high concentrations of mixed sugars, including xylose, glucose, and arabinose. In this study, the reducing sugars in distillers grains could be used as a carbon source by *Rhizopus oryzae* CICC41411 to produce LA. The culture conditions, such as nitrogen source, inoculations size, $CaCO_3$ addition, pH value and fermentation time were found to have a significant effect on the production of LA. Under the optimal conditions ($CaCO_3$ of 80 $g \cdot L^{-1}$, inoculation size of 2.5%, culture pH of 6.0, fermentation time of 96 h), the highest lactic acid concentration (10.88 $g \cdot L^{-1}$) and LA yield (0.53 $g \cdot g^{-1}$ total reducing sugars) was obtained in this study. In comparison with wheat straw hydrolyzate (6.8 $g \cdot L^{-1}$)[2], a relatively high concentration of lactic acid (10.88 $g \cdot L^{-1}$) was produced from DGHs, demonstrating its feasibility of using distillers grains as a novel substrate (mainly carbon source) for LA production from *Rhizopus oryzae*. Efficient production of lactic acid by *Rhizopus oryzae* from DGHs can not only relieve the environmental pollution produced by the brewing industry but also produce valuable products.

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