Optimization of Ethanol Fermentation from Fruit and Vegetable Waste by Plackett-Burman and Orthogonal Experimental Design

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To realize resource technology from fruit and vegetable waste, a Plackett-Burman (P-B) experiment combined with an orthogonal experimental design were adopted for the optimization of ethanol fermentation from this waste. By using the 12-factor P-B design, it was determined that the significant factors were KH_2PO_4, cellulase, and yeast extract. The orthogonal experimental design with the ethanol fermentation and reducing sugar as indices showed that the optimum conditions were KH_2PO_4, cellulase, and yeast extract concentrations of 0.3 g/L, 90 U/mL, and 10 g/L, respectively. Ethanol fermentation from fruit and vegetable waste has provided a feasible application for this waste.

Keywords: Fruit and vegetable waste; Bioethanol; Plackett-Burman

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INTRODUCTION

As dietary structures change amongst people, the ratio of fruit and vegetable waste in city garbage has had a notable upward trend. According to statistics, fruit and vegetable waste generated in China is as high as 1.3 million ton/d, of which 80% is not used and is discarded as rubbish or in the environment (Liu *et al*. 2012a; Shen *et al*. 2013). Because of the high moisture content and biodegradability, conventional landfill treatment not only releases greenhouse gases, but it also produces a large amount of leachate, which has a serious negative impact on the urban environment (El-Fadel *et al*. 2002; Cheng and Hu 2010). As a consequence, there is an urgent need to develop an advanced applicable fruit and vegetable waste disposal technology.

In much of the available renewable energy research, biological fuel ethanol has received a large amount of attention because of its advantages. Because ethanol has a high-octane number, good anti-detonating quality, and CO_2 and H_2O combustion products, and because it can use the characteristics of biomass resources for production, it is considered to be the best environmentally friendly liquid fuel for the future. Therefore, it has clean energy applications (Jensen and Govindan 2014; Asimakopoulos *et al*. 2018; Briand *et al*. 2018; Saravanan *et al*. 2018). Bioethanol accounts for 75% of the biofuel market. China, Brazil and the United States are already using a mixture of bioethanol and gasoline (Panda *et al*. 2018). The energy supply diversification strategy has become a direction for the energy policy of China, in which ethanol is representative of alternative energy sources. Fuel ethanol has broad prospects for development.

The current ethanol fermentation mostly employs food crops, such as corn and other starch sources. These materials increased the production cost (Panda *et al.* 2015). Fruit and

vegetable waste in cities are rich in N, P, and K nutrients and degradable carbohydrates (Panda *et al*. 2016; Ji *et al*. 2017). The research on ethanol fermentation technology using fruit and vegetable waste as raw materials is not only a trend in the energy policy of China, but can also provide reference for the recycling of domestic fruit and vegetable waste. At present, many studies have optimized the process conditions for the fermentation of fruit and vegetable waste to produce ethanol, which has improved processing (Yang *et al*. 2012; Shen *et al*. 2013; Zheng *et al*. 2015). Liu *et al*. (2012b) found that ethanol production had increased after the fruit and vegetable waste had been thermally pre-processed. Wu *et al.* (2017) showed that the pH value can be controlled continuously in continuous ethanol fermentation to achieve stable growth in yield. Huang *et al.* (2015) used a new vacuum fermentation technology to produce more bioethanol at high solids content (35%, w/w). Thammasittirong *et al.* (2013) increased the tolerance of ethanol-producing yeast by random UV-C mutagenesis, which leads to an effective increase in ethanol production. Pavi *et al.* (2017) found that mixed fruit and vegetable waste and municipal solid waste had higher methane production than fruit and vegetable waste.

Based on previous research, the law of ethanol fermentation from fruit and vegetable waste in different seasons was determined. However, considering the convenience of experimentation, raw material fermentation was used directly in previous experiments and optimization of the medium was not done. In this study, a Plackett-Burman (P-B) experiment was combined with an orthogonal experiment and this was used to optimize the composition and conditions of the typical medium for fruit and vegetable waste ethanol production. The goal of this work is to help in the exploration of new resource treatments for fruit and vegetable waste.

EXPERIMENTAL

Materials

Raw materials

The ratio of fruits and vegetables in fruit and vegetable waste was found to be approximately 3:2 based on a previous study. Five kinds of fruits and vegetables were selected in this research. These fruits and vegetables were pears, peaches, apples, potatoes, and beans. Twenty grams of each were used and all of the raw materials were taken from a market in Beijing, China.

Microorganisms and enzymes

Ethanol yeast (Angel Yeast Liability Co., Ltd., Yichang, Hubei Province, China) was used in this experiment. The enzymes included cellulose enzyme (15000 U/g) (Tianjin Guangfu Fine Chemical Research Institute, Tianjin, China), saccharifying enzyme (100000 U/g) (Beijing Aoboxing Biological Technology Co., Ltd., Beijing, China), pectic enzyme (100000 U/g) (Jiangsu RuiYang Biotechnology Co., Ltd., Jiangsu, China), amylase (100000 U/g) (Jiangsu RuiYang Biotechnology Co., Ltd.), inorganic salt (KH_2PO_4), *etc.*

Methods

Fermentation experiments

First, 0.1 g of yeast was added to 100 g of crushed fruit and vegetable waste as an inoculum. The amount of enzyme preparation was set according to the experimental table, and fermentation was performed after adding an appropriate amount of nutrient salt. It was

placed in an Erlenmeyer flask for sealed anaerobic fermentation, and the entire process was carried out in a shaker with the speed of 145 r/min. The experimental temperature and pH were set to 37 °C and 6, separately. The experimental period was from 48 h to 60 h.

P-B experiment

The P-B design method is a two-level experimental design method developed in the mid to late 20th century. Based on the principle of a non-completely balanced block, it can test multiple independent adjustable variables in one experiment and select the factors that have significant influence on the experimental results through statistical analysis. The method is effective and accurate at selecting important factors and therefore, it is widely used in the screening of microbial culture media.

Table 1 shows the 11 factors for the 12 experiment groups that were designed. The enzymes included pectinase, saccharifying enzyme, cellulase, amylase, and protease. The nitrogen source was yeast extract, and the inorganic salts were $MgSO_4$, KH_2PO_4, $CaCl_2$, $(NH_4)_2SO_4$, and a blank.

Table 1. Plackett-Burman Design Factors and Levels

Serial Number	Factor	Level of Factor	
		Low-level (-1)	High-level (+1)
A	Pectinase (U/g)	0	100
B	Saccharifying Enzyme (U/g)	0	100
C	Cellulase (U/g)	0	50
D	Amylase (U/g)	0	50
E	Protease (g/L)	0	100
F	Yeast Extract (g/L)	0	27.1
G	$MgSO_4$ (g/L)	0	6.0
H	KH_2PO_4 (g/L)	0	0.3
I	$CaCl_2$ (g/L)	0	5.0
J	Blank	-	-
K	$(NH_4)_2SO_4$ (mg/L)	0	6.0

The dose for the enzyme is based on the activity per fermentation broth mass, the concentration for different salt is based on the concentration per volume of fermentation broth.

Table 2. Factor and Level of the Orthogonal Design Experiment

Number	Cellulase (U/g)	Yeast Extract (g/L)	KH_2PO_4 (g/L)	Blank
1	30	10	0.3	0
2	30	30	0.6	0
3	30	50	0.9	0
4	60	10	0.6	0
5	60	30	0.9	0
6	60	50	0.3	0
7	90	10	0.9	0
8	90	30	0.3	0
9	90	50	0.6	0

Orthogonal experiment

The orthogonal experimental design is another design method used to study multi-factor and multi-level variables. It is based on orthogonality to select some representative points from the comprehensive experiment. These representative points are evenly

dispersed and comparable. This study used the L_9 (3^4) design, and the relevant design is shown in Table 2.

Analysis method

A certain volume of fermented broth was taken, centrifuged at 10000 rpm for 15 min, and the supernatant was used to determine the reducing sugar and ethanol contents. The DNS method was used for the reducing sugar content, and the ethanol content was determined by SBA-40c (Academy of Sciences Institute of Shandong Province, China). The analysis of the P-B experiment was performed with Statistica 6.0 (StatSoft, USA), and the orthogonal experiment was performed with SPSS-19.0 (IBM, USA).

RESULTS AND DISCUSSION

P-B Design Method for Screening Important Factors

According to the design in Table 1, the nutrient elements were added to the basic medium. The ethanol fermentation was done according to the method given in the Experimental section, and the content of ethanol produced is shown in Table 3. Table 4 shows the experimental analysis and the *p* value was used to determine the factors with a significant influence at a 95% confidence interval ($p < 0.05$). The *p*(a) values in Table 4 showed that KH_2PO_4 corresponded to the smallest *p* value of 0.051.

Among the variables examined, the cellulase, yeast extract, and KH_2PO_4 were significant factors ($p < 0.1$). The order of significance was as follows: KH_2PO_4 > cellulase > yeast extract. Phosphate had a significant effect on increasing the ethanol production. This was consistent with the results of Janke *et al.* (2017), who showed that adding phosphate resulted in faster degradation kinetics and higher production when fermenting sugarcane straw.

Vintilă *et al.* (2015) showed that the addition of phosphorus (KH_2PO_4) increased ethanol production in sweet sorghum juice fermentation. Therefore, during ethanol fermentation from fruit and vegetable waste, phosphorus as a nutrient element may accelerate the degradation kinetics and promote the production of ethanol.

The effect of cellulase on the experiment was significant. Because of the rich cellulose content in vegetables and peels, the addition of cellulase could increase the utilization of raw materials during ethanol fermentation. The addition of yeast extract not only provided a nitrogen source, but also contained some minerals. Yeast extract could meet the needs of microbial metabolism and increase their activity. Therefore, it had a significant impact on ethanol fermentation.

Nawaz *et al.* (2018) also confirmed that a medium containing yeast extract had the highest enzyme activity compared with other inorganic nitrogen sources. Also, the yeast extract quality was stable and cost-effective, which is the basic choice for general industrial fermentation.

The type of nutrient elements was determined from the results of the P-B experiment. To optimize the composition of the medium, the content of the respective nutrient elements should also be confirmed. Therefore, a corresponding experimental analysis should be performed.

Table 3. Results of the P-B Experimental Design

Number	A	B	C	D	E	F	G	H	I	J	K	Ethanol Content (g/L)
1	+1	-1	+1	-1	-1	-1	+1	+1	+1	+1	-1	11.0±0.77
2	+1	+1	-1	+1	-1	-1	-1	+1	+1	-1	+1	13.4±0.72
3	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1	14.4±1.55
4	+1	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1	16.0±1.40
5	+1	+1	-1	+1	+1	-1	+1	-1	-1	+1	-1	21.8±1.31
6	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	16.2±1.39
7	-1	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	19.8±1.08
8	-1	+1	+1	+1	+1	-1	+1	+1	-1	-1	+1	7.0± 0.38
9	-1	-1	-1	+1	+1	+1	-1	+1	+1	+1	-1	18.4±1.63
10	+1	-1	-1	-1	+1	+1	+1	-1	+1	-1	+1	25.2±0.74
11	-1	-1	-1	-1	-1	+1	+1	+1	-1	+1	+1	13.2±0.71
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	18.0±1.12

Table 4. Analysis of the P-B Design for Ethanol Fermentation

Serial Number	Factor	*p*(a)
A	Pectinase (U/g)	0.140
B	Saccharifying Enzyme (U/g)	0.330
C	**Cellulase (U/g)**	**0.071**
D	Amylase (U/g)	0.436
E	Protease (g/L)	0.206
F	**Yeast Extract (g/L)**	**0.078**
G	$MgSO_4$ (g/L)	0.675
H	**KH_2PO_4 (g/L)**	**0.051**
I	$CaCl_2$ (g/L)	0.177
J	Blank	
K	$(NH_4)_2SO_4$ (mg/L)	0.113

Analysis of the Orthogonal Experiment Results

Table 5. Result of the Orthogonal Experimental Design

Number	Cellulase	Yeast Extract	KH_2PO_4 (g/L)	Blank	Ethanol (g/L)	Reducing Sugar (g/L)
1	30	10	0.3	0	26.4±0.88	0.181±0.081
2	30	30	0.6	0	27.0±0.90	0.253±0.023
3	30	50	0.9	0	25.4±1.48	0.155±0.017
4	60	10	0.6	0	26.4±3.21	0.138±0.014
5	60	30	0.9	0	26.2±1.53	0.104±0.010
6	60	50	0.3	0	37.0±2.23	0.319±0.029
7	90	10	0.9	0	35.8±1.80	0.132±0.015
8	90	30	0.3	0	30.8±2.73	0.146±0.017
9	90	50	0.6	0	23.4±0.68	0.137±0.006

The orthogonal experiments were used to analyze three factors with significant effects. The results are shown in Table 5. To fully consider the effects of the ethanol and reducing sugar contents, a dual-index analysis was used. Their respective variances and salience are shown in Table 6. The results were analyzed by an analysis of variance.

Table 6. Analysis of the Orthogonal Experimental Results

Source	F(ethanol)	*p*(ethanol)	F(sugar)	*p*(sugar)
Cellulase	0.267	0.789	0.349	0.741
Yeast Extract	0.036	0.966	0.266	0.790
KH_2PO_4	0.509	0.663	0.651	0.606

It was assumed that the factor with a *p* value less than 0.05 was significant. However, Table 6 shows that the three factors selected were not significant factors for ethanol and reducing sugar production. Mathematically, these factors were not important, but they were still of crucial importance for ethanol fermentation. Inei-Shizukawa *et al.* (2009) found that the optimum zeolite concentration during ethanol fermentation was 0.2 g/L. Because of the similar concentration, the zeolite addition was not as important as the other factors that had significant effects. However, the optimization of this culture condition resulted in a 20% increase in the ethanol production, which indicated the importance of zeolite in ethanol optimization research. In this study, the significance of the three factors was as follows: KH_2PO_4 > cellulase > yeast extract. According to the optimum conditions, it was determined that for ethanol production, the optimum conditions were 90 U/mL cellulase, 30 g/L yeast extract, and 0.3 g/L KH_2PO_4 (shown in S1, differential map). To fully consider the experimental process, the effect on the reducing sugar concentration was also determined. Table 6 shows that the corresponding factors were also insignificant

factors, but the order of influence of each was as follows: KH_2PO_4 > cellulase > yeast extract. As is shown in S2, the optimum conditions determined were 90 U/mL cellulase, 10 g/L yeast extract, and 0.3 g/L KH_2PO_4. Under these experimental conditions, the reducing sugar concentration was the lowest.

Considering the levels of the two indicators, it was observed that the optimum levels for the cellulase and KH_2PO_4 were consistent. For different yeast extract contents, the best condition was 10 g/L from an economic point of view. Therefore, the final optimum conditions were as follows: 90 U/mL cellulase, 10 g/L yeast extract, and 0.3 g/L KH_2PO_4.

The average TS in this experiment was determined to be 12%, and the highest ethanol concentration is 37 g/L. Since 100 g fermentation broth was used in this study, and the density is about the same with water, with the calculation we could get the ethanol yield 0.308 g ethanol /g TS.

CONCLUSIONS

1. Plackett-Burman design and orthogonal experiment principles were applied in this study to optimize the ethanol fermentation from fruit and vegetable waste. The influential parameters among 11 factors were determined to be cellulase, yeast extract and KH_2PO_4.
2. The corresponding optimum conditions optimized by orthogonal experimental design based on ethanol concentration and reducing sugar showed that a maximum ethanol concentration of 37 g/L could be achieved with the conditions of cellulase 90 U/g, yeast extract 10 g/L, and KH_2PO_4 0.3 g/L. The corresponding optimum ethanol concentration was 0.308 g ethanol/g TS.
3. Such technology can utilize fruit and vegetable waste and achieve ethanol as a product, thus attaining the dual goals of environmental protection and resource recovery.

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