Ethanol Production from Kitchen Garbage by *Zymomonas mobilis*: Optimization of Parameters through Statistical Experimental Designs

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Plackett-Burman design was employed to screen 8 parameters for ethanol production from kitchen garbage by *Zymomonas mobilis* in simultaneous saccharification and fermentation. The parameters were divided into two parts, four kinds of enzymes and supplementation nutrients. The result indicated that the nutrient inside kitchen garbage could meet the requirement of ethanol production without supplementation, only protease and glucoamylase were needed to accelerate the ethanol production. The optimum usages for both enzymes were determined to be A = 100 U g^{-1} by single factor experiment. Then the parameters including initial pH, time and temperature were optimized during the fermentation by using central composite experimental design (CCD). The results of second-order polynomial model indicated that interactions between the factors showed no crucial effect on ethanol production. The optimum conditions were determined to be initial pH of 4.95, time of t = 30.69 h, temperature of θ = 31.22 °C, the corresponding maximum ethanol was γ = 53.20 g L^{-1}. Ethanol production from kitchen garbage enjoyed the advantages of simple process, low cost and short fermentation time, which should be further studied to make it applicable.

Key words:
Zymomonas mobilis, ethanol fermentation, kitchen garbage, Plackett-Burman experimental design, central composite experimental design

Introduction

Over the last century, energy consumption has increased tremendously due to the growth of world population and industrialization.[1] Interest in the utilization of renewable carbohydrate sources to produce fuel ethanol as an alternative to petroleum is rising around the world to save petroleum and natural gas.[2–4] The ideal raw materials for ethanol production should be cheap and rich in nutrient. Some renewable feedstock such as sago starch, cellulose materials have been investigated.[5–7] Few researches are carried out on the utilization of kitchen garbage. Being a kind of municipal waste with high volume, kitchen garbage is difficult to be handled owning to its high organic content and moisture. On the other hand, it could also be regarded as a valuable resource due to its content of nutrients. It is reported that hydrogen, lactic acid and other substances could be produced from it.[8–11] The research taken on by Li[12] adopted the organic solid based on European standard to produce ethanol. The raw materials were a mixture of waste from kitchen garbage and yard waste. Little information showed the utilization of Chinese kitchen garbage, since Chinese have a unique eating habit, the investigation on Chinese kitchen garbage to ethanol could provide valuable information for the recycling technology.

During the fermentation process, suitable production medium and variables optimization are of crucial importance. For ethanol production, researchers have paid much interest in utilizing the *Zymomonas mobilis.* This kind of facultative anaerobic bacterium has been extensively studied over the last decade due to its efficient production of ethanol from sucrose and fructose.[13–15] In order to obtain the maximum yield of ethanol with complete utilization of the substrate to reduce pollution encountered in the spent broth, suitable medium optimization should be carried out. Medium optimization by single factor experiment is laborious and time consuming. Thus the statistical methodology was adopted to optimize the nutrients for the medium to acquire the optimum ethanol yield. Several statistical factorial designs ranging from two-facto-

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rial to multi-factorial designs are available for optimization. Among them, the Plackett-Burman (P–B) statistical method could study n variables in n+1 experimental runs, which means it could screen variables with less time, chemical and manpower. Furthermore, since the design is orthogonal in nature, implying that effect of each variable would not interfere with each other. Thus the P–B design could be a useful tool in optimizing the medium for fermentation.[16–20]

Another study of the fermentation process involves the optimization of culture parameters. Similar to the nutrient screening process, utilization of statistical designs could get rid of the shortcomings of the traditional single factor experiments. Moreover, it could reveal the relationship between the variables which is of great importance during the fermentation process. Among them, response surface methodology (RSM) is one suitable method for identifying the effect of individual variables and for seeking the optimum conditions for a multivariable system efficiently. This process has been successfully applied to optimize ethanol fermentation and other product fermentation.[21–22] The utilization of this method in this study could determine the relations between the factors as well as the optimal ethanol production conditions.

The aim of this study is to adopt P–B design to optimize the medium composition for ethanol production from kitchen garbage. Different kinds of ingredients were tested for their effect on ethanol production. The CCD design was applied to reveal the relations between physical parameters such as initial pH, temperature and time, the determination of corresponding optimum conditions would also be investigated.

Materials and methods

Strain and medium

Zymomonas mobilis 10225 was obtained from China Center of Industrial Culture Collection. The strain was maintained on agar slants having composition (γ/g L^{-1}): glucose 100, yeast extract 10, KH_2PO_4 1, $(NH_4)_2SO_4$ 1, $MgSO_4$ 0.5. The microorganism was grown at a temperature of θ = 30 °C and pH 5.5. It was cultured for t = 48 h before its application in the fermentation.

Fermentation of kitchen garbage

Kitchen garbage was collected from the dining room of the University of Science and Technology Beijing. The garbage was ground and mixed completely, then put into the refrigerator under θ = –20 °C for future use. During the fermentation experiment, m = 100 g of smashed kitchen garbage mixed with V = 50 mL water were put into a conical bottle. Then the bottle was placed in a constant-temperature shaker (MH-86 Guo Hua Company, China). Except the specified conditions mentioned, initial pH was controlled to 5.5 by dilute acid c_{HCl} = 0.05 mol L^{-1} or base c_{NaOH} = 0.05 mol L^{-1}, time and temperature were kept at t = 48 h and θ = 30 °C respectively. Inoculum size was maintained to be 10 % (V/m) other substrates should add according to the experimental design.

Kinds of enzyme utilized in this study

Cellulase, α-amylase, protease and glucoamylase were obtained from Beijing Dong Hua Qiang Sheng Biochemical Technology Company. The enzyme activities were A = 5000 U g^{-1}, 10000 U g^{-1}, 50000 U g^{-1}, 100000 U g^{-1} respectively.

Analytical methods

The levels of total solid (TS), suspended solid (SS) and dissolved solid (DS) for the kitchen garbage were measured according to the standard procedure described in APHA and Wang.[23–24] The detail procedure was as followed: Kitchen garbage (m_1/g), was dried to stable mass at 100–105 °C, mass (m_2/g). The kitchen garbage (m_1/g) was filtered to obtain leached residue (m_3/g) and leached liquid (m_4/g). Leached residue was dried to stable mass (m_5/g), while liquid was dried to stable mass (m_6/g). Then w_{TS}, w_{SS} and w_{DS} were defined as:

$$w_{TS} = \frac{m_2}{m_1} \cdot 100 \quad w_{SS} = \frac{m_5}{m_1} \cdot 100 \quad w_{DS} = \frac{m_6}{m_1} \cdot 100$$

In addition, each sample was filtered through a 0.45 μm-pore membrane after being centrifuged at 4000 rpm for t = 30 min at θ = 4 °C. The filtrate was subject to analyses of ethanol concentration and soluble sugar. Ethanol was quantified by gas liquid chromatography (Flame Ionization Detector, GC16 Shimadzu, Kyoto) by the method of Anthony.[25] Sugars were determined according to Miller's method.[26]

Plackett-Burman experimental design

The P–B design was used to identify which variables have significant effects on ethanol production by *Z. mobilis* 10225. The 12-run P–B design included enzymes and nutrients. Among the enzymes, α-amylase, glucoamylase, cellulase and protease were tested for their effects on the ethanol production. Furthermore, nitrogen source and inorganic salts were investigated to reveal their influence on ethanol production. The experimental de-

Table 1 – *Variables screened in PB design and their real values*

	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8
	α-amylase A/U g^{-1}	glucoamylase A/U g^{-1}	cellulase A/U g^{-1}	protease A/U g^{-1}	KH_2PO_4 γ/g L^{-1}	$MgSO_4$ γ/g L^{-1}	$(NH_4)_2SO_4$ γ/g L^{-1}	yeast extract γ/g L^{-1}
High level (+1)	50	100	120	100	1	0.5	1	5
Low Level (-1)	0	0	0	0	0	0	0	0

Table 2 – *Plackett-Burman design for 8 variables with observed results for ethanol production in SSF*

X_1	X_2	D_1	X_3	X_4	D_2	X_5	X_6	D_3	X_7	X_8	Ethanol γ/g L^{-1}
1	–1	1	–1	–1	–1	1	1	1	–1	1	13.25
1	1	–1	1	–1	–1	–1	1	1	1	–1	42.88
–1	1	1	–1	1	–1	–1	–1	1	1	1	44.85
1	–1	1	1	–1	1	–1	–1	–1	1	1	13.26
1	1	–1	1	1	–1	1	–1	–1	–1	1	45.08
1	1	1	–1	1	1	–1	1	–1	–1	–1	49.89
–1	1	1	1	–1	1	1	–1	1	–1	–1	48.65
–1	–1	1	1	1	–1	1	1	–1	1	–1	40.56
–1	–1	–1	1	1	1	–1	1	1	–1	1	45.12
1	–1	–1	–1	1	1	1	–1	1	1	–1	34.89
–1	1	–1	–1	–1	1	1	1	–1	1	1	45.05
–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	12.98

sign are shown in Tables 1 and 2. Eight variables ($X_1 - X_8$) – i.e. α-amylase, glucoamylase, cellulase, protease, KH_2PO_4, $MgSO_4$, $(NH_4)_2SO_4$ and yeast extract were chosen as the candidate factors, while D_1, D_2, D_3 were dummy factors employed to evaluate the standard errors of the experiment. Low levels (–1) and high levels (+1) were assigned for each factor. The average values of ethanol concentration were used as the response in this design (all the experiments were repeated three times). The significance of variables was determined by student's test. Variables with $p < 0.05$ were considered significant.

Central composite experimental design

Central composite design (CCD) was used in the optimization of ethanol production. Time (X_1/h), pH (X_2), temperature (X_3/ °C) were chosen as the independent variables shown in Table 3. Ethanol concentration (γ/g L^{-1}) was used as dependent output variables. Twenty experiments were performed according to Table 4 to optimize the parameters. Among them, six replications were at center points ($n_0 = 6$), while the axial points were determined to be $\sqrt{3}$. The coefficients of the polynomial model were calculated using the following equation:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ij} X_i^2 + \sum_{i_{<j}}^{k} \sum_{j}^{k} b_{ij} X_i X_j$$

Where Y is predicted response, and i, j are linear, quadratic coefficients, respectively. b and k are regression coefficients and the number of factors studied in the experiment, respectively.

The significance of each coefficient was determined using student's value. The results were analyzed by Statistica 6.0 (StatSoft Company, USA).

Table 3 – *Variables in the CCD design*

Variables	Coded levels				
	–1.682	–1	0	1	1.682
time, t/h	13.2	20	30	40	46.8
pH	3.4	4	5	6	6.6
temperature, θ/°C	22.6	25	30	35	38.4

Table 4 – *CCD experiment for optimizing ethanol yield using Z. mobills*

Time t/h	Initial pH	Temperature θ/°C	Ethanol γ/g L^{-1}
20	4	25	6.83
20	6	35	14.18
40	4	35	19.97
40	6	25	10.84
30	5	30	51.54
30	5	30	51.94
20	4	35	16.75
20	6	25	6.44
40	4	25	10.1
40	6	35	21.86
30	5	30	52.64
30	5	30	51.99
13.2	5	30	5.84
46.8	5	30	13.04
30	3.4	30	39.4
30	6.6	30	34.39
30	5	22.6	10.76
30	5	38.4	32.15
30	5	30	52.64
30	5	30	51.95

Table 5 – *Characteristics of kitchen garbage used in the experiment*

Components	Mass fraction w/%	Average, w/%
w_{TS}	11.97 ~ 18.10	17.22
w_{DS}	1.31 ~ 4.96	2.58
w_{SS}	7.01 ~ 15.80	14.64
total sugar	56.78 ~ 67.10	62.68
starch	41.38 ~ 55.09	46.12
protein	13.21 ~ 17.13	15.56
fat	15.12 ~ 19.89	18.06
cellulose	1.91 ~ 2.87	2.26

The number was calculated based on dry mass

Table 6 – *Estimate of coefficient of factors and associated significant levels for P–B design*

Variables	Coefficient	t	p
mean	36.16667	15.68789	0.000563
α-amylase	–3.33333	–1.44589	0.243982
glucoamylase	9.66667	4.19308	0.02474
cellulase	3	1.3013	0.284077
protease	6.83333	2.96407	0.05935
KH_2PO_4	1.66667	0.72294	0.521979
$MgSO_4$	2.83333	1.229	0.306679
$(NH_4)_2SO_4$	0.66667	0.28918	0.791278
yeast extract	–1.83333	–0.79524	0.484582

Three-dimensional plots and their respective contour plots were obtained to study the interaction of one parameter with another. The optimum concentration was identified based on the hump in the three dimensional plots.

Results and discussion

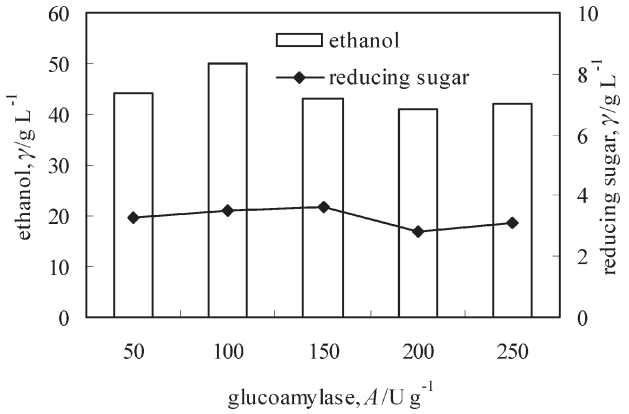
Determination of significant factors affecting ethanol production from kitchen garbage

Table 5 shows the characteristics of kitchen waste. These characteristics indicate that the kitchen waste is mainly composed of sugar, protein fat and cellulose. It showed that it could be regarded as a suitable substrate for ethanol production. To further optimize the culture medium, P–B design was utilized to determine the affecting factors for the experiment. Table 6 shows the resulting effects of the variables on the responses and the associated t-values and significant levels. Among the four enzymes, the glucoamylase and protease were determined as significant, followed by cellulase and α-amylase. Generally, α-amylase should be very important in ethanol production from starchy substrates, because it could liquefy the substrate and accelerate the hydrolysis of starch. The difference in this experiment might be the special property of the kitchen garbage. Since most kitchen garbage underwent thermal treatment in cooking, such process could shorten the chain of the starch, then the glucoamylase could utilize these substances to produce glucose. Thus, glucoamylase showed crucial importance during the process, while α-amylase showed trivial effect on the process. The protease also showed comparatively important effect (p = 0.059350). It might be because the protein could be beneficial for the growth of the organism. Furthermore, it could help dissolve the substrate and release more soluble sugar.[27] When it came to cellulase, the low significance might be because the kitchen garbage contained low concentration of cellulosic substrates, thus cellulose was not needed. Thus, different sources of kitchen garbage should utilize different enzymes to obtain the highest yield.

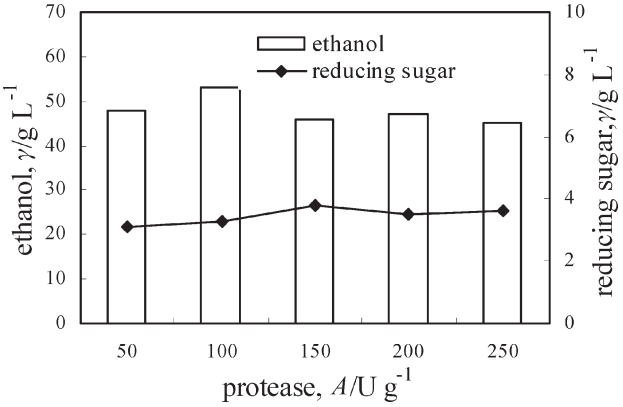
As for the inorganic salts, none of the tested items showed importance. That does not mean that these substrates had a trivial effect on ethanol production. The reason was that the abundant nutrition in kitchen garbage could meet the requirement of the organism. From this point of view, kitchen garbage is a suitable source for ethanol production, it could utilize two kinds of enzymes to produce ethanol without adding extra nutrition. Such process could simplify the technology and decrease the cost.

The P–B design showed that the protease and glucoamylase were of great importance to the ethanol production from kitchen garbage. Then the optimum usage of the enzyme were determined by the following experiment. They were carried out according to the procedure mentioned in 2.2. Fig. 1 shows that the ethanol was the highest at A = 100 U g^{-1} glucoamylase. When the usage was higher than A = 100 U g^{-1}, the more enzymes used, the lower the ethanol produced. This was probably because the sugar released at high concentration of enzymes could not be utilized promptly by the bacteria, and high sugar concentration would have a negative effect on bacteria. Furthermore, the abundant sugar at initial stage would cause quick growth of microorganism, fast consumption of the sugar and severe competition between microorganisms in time of less sugar, thus proper enzyme usage would benefit ethanol production. When the reducing sugar was considered, the low concentration under all these conditions demonstrated that the substrate was mostly consumed in the end. Taking ethanol production cost into consideration, the enzyme usage for glucoamylase was determined to be A = 100 U g^{-1}.

As shown in Fig. 2, the concentrations of reducing sugars under different protease usage were all very low, which showed that the substrate could be well utilized. Regarding ethanol production, the highest concentration was achieved at a protease



F i g . 1 – *Effect of glucoamylase usage on ethanol production (with glucoamylase added only)*



F i g . 2 – *Effect of protease usage on ethanol production (with glucoamylase 100 U g^{-1} added) □ ethanol ◆ reducing sugar*

usage of A = 100 U g^{-1}. Further increases of protease activity led to a decrease of ethanol; the reason might be that the high concentration nutrients released with the help of protease caused high competition between organisms. Thus, the proper usage of enzyme would benefit ethanol production. Furthermore, the protease could shorten the experiment time since it could release the nutrient as well as decrease the viscosity of the substrates.[28] The optimum time should be further investigated in the following experiment.

Optimization of fermentation parameters using Surface Response Methodology

In order to reveal the significant physical factors that affected the fermentation process, the experiment was analyzed by software (Statistica 6.0). Table 7 shows the regression coefficients and significance levels (coefficients a). The items underlined were proved significant ($p < 0.05$), from the result we could see that the interactions between the factors showed no crucial effect on ethanol production. In order to simplify the model, the item that showed trivial effect on the model was omitted. After the data were reevaluated by the software, the coefficients showed a slight difference in the new model (Table 7 coefficients b). All the items showed important effect on ethanol production. ($p < 0.01$). The second order polynomial equation giving the ethanol as a function of time (13.2 h < X_1 < 46.8 h), pH (3.4 < X_2 < 6.6), temperature (22.6 °C < X_3 < 38.4 °C) was obtained as followed:

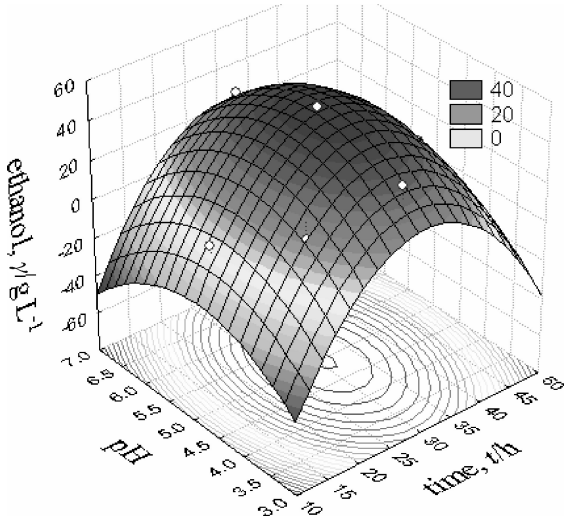
$$Y = -806.347 + 9.926\,X_1 + 71.488\,X_2 + 33.953\,X_3 - \\ - 0.162\,X_1^2 - 7.213\,X_2^2 - 0.544\,X_3^2$$

The R^2 was determined to be 0.9758, which showed the model was quite a good fit. Figs. 3–5 show the effect of time, pH and temperature on the ethanol production. The optimal value of each fac-

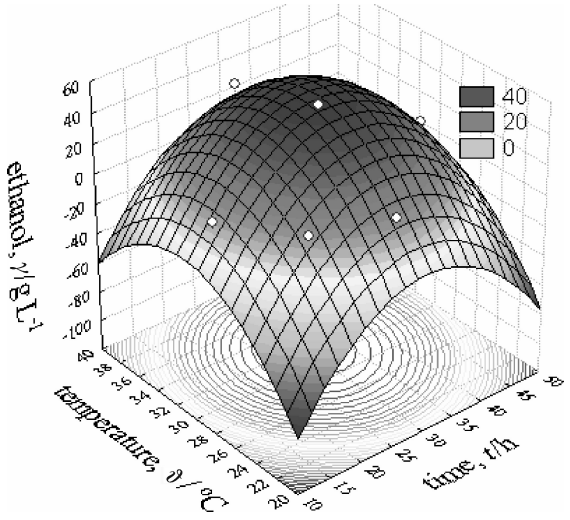
T a b l e 7 – *Estimate of coefficients of factors and associated significant levels for CCD*

	Regression coefficient (*a*)	*p*	Regression coefficient (*b*)	*p*
mean	–792.471	0.000001	–806.347	0
time	9.334	0.000022	9.926	0
time*time	–0.162	0	–0.162	0
pH	70.168	0.000713	71.488	0.000008
pH*pH	–7.213	0.000073	–7.213	0.000007
temperature	33.84	0.000001	33.953	0
temperature* temperature	–0.544	0	–0.544	0
time*pH	0.07	0.626301		
time*temperature	0.008	0.7772		
pH*temperature	–0.026	0.927776		

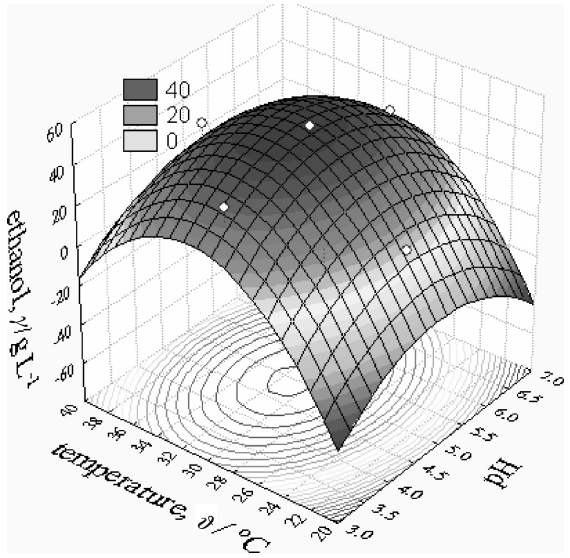
tor is also clearly shown in the plots. Fig. 3 shows the time and pH of ethanol production. Ethanol reached a maximum value at about t = 30 h and pH 5. The ethanol biosynthesis as the function of time and temperature are shown in Fig. 4. The optimum values of time and temperature are close to central point. Fig. 5 also shows that the optimal conditions for ethanol production were pH 5 and temperature of θ = 30 °C. The optimal conditions for ethanol production were obtained by further numerical analysis of the response surface using Statistica 6.0 software. The solution to the maximal ethanol was 30.69 h for time, initial pH 4.95, θ = 31.22 °C, and the ethanol was γ = 53.20 g L^{-1}. A verification experiment under the condition mentioned above was conducted in order to confirm the optimal conditions obtained from the statistically based experimental design. The result was proved to be γ = 52.50 g L^{-1}, which was very close to the predicted value of γ = 53.20 g L^{-1}. Therefore, the model was useful to predict the concentration as well as the optimization of the experimental conditions. The industrial application usually adopts active dry *Saccharomyces cerevisiae* as the seed to produce ethanol, the average time should be over t = 60 h, the utilization of *Z. mobilis* could reach the final point at about t = 30 h, such short processing time was of crucial importance in industrial application. The advantage of low experimental time has been realized in this study, and further research should be carried out to improve the ethanol yield to make this technology more successful in practice.



F i g . 3 – *Response surface and contour plot of time vs. pH on ethanol production*



F i g . 4 – *Response surface and contour plot of temperature vs. time on ethanol production*



F i g . 5 – *Response surface and contour plot of temperature vs. pH on ethanol production*

Conclusion

Kitchen garbage was used to produce ethanol by *Zymomonas mobilis* using statistically based experimental design. Plackett-Burman design was used to identify which variables had significant effect on ethanol production. The results showed that the abundant nutrition in kitchen garbage made it feasible to produce ethanol without adding extra substrate. Only glucoamylase and protease were needed to increase the ethanol yield. The optimum usages for both these enzymes were determined at A = 100 U g^{-1}. The parameters including pH, time and temperature were optimized during the fermentation by using CCD design. The optimum conditions were determined at t = 30.69 h, pH of 4.95, temperature of θ = 31.22 °C, the corresponding ethanol was γ = 53.20 g L^{-1}.

Comparing the conventional and time-consuming one-variable-at-a time approach, the statistically based experimental designs proved efficient tools in this optimization process. Further research should be carried out to make the technology more efficient and cost-competitive. Thus, kitchen garbage could be regarded as a valuable resource for ethanol production.

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List of symbols

A – enzyme activity, U g^{-1}
a, b – regression coefficients
c – concentration, mol L^{-1}
k – number of factors
m – mass, g
n – number of variables
p – level of significance
R^2 – determination coefficient
t – time, h
V – volume, mL, L
w – mass fraction, %
X – coded variable
Y – predicted factor
γ – mass concentration, g L^{-1}
θ – temperature, °C

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