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ORIGINAL ARTICLE

Factorial Analysis on Biovinegar Production from Pineapple Waste Using Mixed Strains

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ABSTRACT – One of the feasible approaches to oversee pineapple waste deposit without harming the environment is by converting these build-ups into value added items such as biovinegar. The objective of this work is to screen the fermentation parameter to identify the best condition and significant parameters affecting the fermentation. Five independent parameters were investigated, namely; temperature, fermentation time, addition of glucose, part and condition of waste. Fractional factorial design of Design Expert® software was used to investigate the effect of independent parameters as well as the interaction between parameters on the biovinegar production. The work was carried out by natural fermentation in which naturally occurred microorganism readily available on the raw materials (pineapple waste) was used. The result showed that the order of parameter significance in acid production was as follows: temperature > addition of glucose > fermentation time > part of waste > condition of waste. The interaction parameter of fermentation time and addition of glucose had the strongest effect on the acid production. The best fermentation condition was carried out using pineapple peel juice at 30 °C for 8 days in an anaerobic condition with 50 g/L glucose addition. Under these conditions, acid production was 1.12 % w/v in which acetic acid concentration was 0.94 % w/v. The product pH was recorded at 3.57. The product yield and productivity were recorded at 0.1699 g/g and 0.0489 g/L.h, respectively. Exploration on producing biovinegar using mixed strains and pineapple waste as substrate could be another way to reduce environmental pollution and at the same time turning this waste into value added product. Moreover, using the natural fermentation together with the carry over benefit of the pineapple benefitted the quality of produced biovinegar.

KEYWORDS
Biovinegar, mixed strains, fractional factorial design, pineapple waste, natural fermentation.

INTRODUCTION

During pineapple processing, the stem, crown, core and peel were removed and discarded as waste. As much as 50 % of the total fruit weight were discarded as waste during canning (Salim, 2016). It can be estimated that more than 150, 000 kg of pineapple waste was produced each year in Malaysia. This waste disposal can be problematic because it is high in moisture and sugar content and prone to microbial spoilage emitting foul gaseous such as H_2, CO_2 and CH_4 (Lun et al., 2014).

Usually, biovinegar production utilizes specific microorganism strain for the fermentation to occur. However, it has been reported that biovinegar from single strain fermentation is inferior to that from the mixed strains (Liu et al., 2019). The use of mixed strain together with the carry over benefit of the raw materials were seen to improve the aroma and quality of biovinegar (Liu et al., 2019). Study by Liu et al. (2019) and Chen et al. (2017) proved that mixed strains apple and citrus biovinegar were rich in flavour, aroma and antioxidant activity than its single strain. The natural occurring microorganism in the pineapple waste were identified from Monera and Fungi kingdom with various genus such as *Pseudomonas*, *Erwinia*, *Xanthomonas*, *Acidovorax* and *Acetobacter* for the bacteria species, meanwhile, *Penicillium*, *Geotrichum*, *Fusarium*, *Botrytis*, *Collectotrichum*, *Muco*, *Monilinia*, *Rhizopus* and *Phtyophthora* for fungi species (Bhat et al., 2014).

Previous study on pineapple biovinegar focused on one factor at a time (OFAT) investigation in which much time was needed to complete if a lot of parameters were considered (Raji et al., 2012). The data analysis also limited to only one parameter contribution instead of parameter interaction. No study has been reported on multiple parameters using statistical tool such as fractional factorial design on the biovinegar production.

Temperature is one of the important parameters to be investigated as it would affect greatly on the growth of microorganism thus directly affecting the acid production. Study by Ghosh et al. (2014), Chakraborty et al. (2017a) and Kong et al. (2018) reported a temperature range between 30 and 32 °C was optimum for the growth of *Acetobacter* and yeast. Meanwhile, Arroyo-López et al. (2009) found that a special thermotolerant *Acetobacter* was able to sustain its

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growth at elevated temperature of 40 °C. Thus, for the screening study, the range of parameter should be wider than the reported one, hence the chosen temperature was between 30 and 50 °C. Fermentation time is another important parameter that should be considered to ensure the alcohol and acid production at sufficient and appropriate track of time. Previous study stated that it took between 11 to 40 days for the alcoholic and acidic fermentation to complete (Raji et al., 2012; Roda et al., 2017). Carbon source is another important element for continued growth of microorganism. Raji et al. (2012) carried out a fermentation using pineapple peel with the addition of 25 g/L glucose, thus it was decided to apply a wide range of glucose addition in this screening study which was between 0 and 50 g/L.

The objective of this work is to screen the fermentation parameter to identify the best condition and significant parameters affecting the fermentation. Factorial analysis was performed by using two level factorial of Design Expert® software. Two level factorial is a statistical method based on multivariate non – linear model that is useful in studying interactions of various parameters affecting the process (Bergquist, 2015; Saunders & Eccleston, 1992). The fermentation kinetics including product yield and its productivity was also developed. These were determined using the best conditions suggested by the software.

MATERIALS AND METHODS

Raw materials

The pineapple fruits used for this study were from MD2 species and was provided generously by Pekan Pina Sdn. Bhd. The fruits were cleaned and cut to separate the peel and core. Cleaned peel and core were pureed to produce a slurry. The juice substrate was prepared by extracting the slurry through a filter with pore size of 20 µm. All substrate was kept at -20 °C until further use.

Experimental design

In this work, five parameters which were fermentation time, temperature, addition of glucose, condition and part of waste were taken into account to investigate their effects on the percentage of acid production using 2^{5-1} fractional factorial design produced 16 runs of experiments. The design of experiment was performed by Design Expert® software where all parameters were randomized. Table 1 shows the design parameters and levels were coded as -1 (low level) and +1 (high level) where low and high levels indicates the lowest and the highest range of the parameters. Batch natural fermentation without agitation was carried out anaerobically using pineapple waste substrate with the readily available microorganism on the substrate itself. Each fermentation run was conducted in a 100 mL serum bottle with 50 mL working volume. Once fermentation ceased, sample was collected and centrifuged at 8000 rpm for 15 minutes and subjected to analysis. Carried out analysis were pH, acid content, acetic acid concentration and reducing sugar concentration. The response of the experimental design was analyzed using ANOVA based on the *p*-value with 95 % of confidence level. Experimental data was analyzed to determine the percentage contribution of all parameters and interaction between them.

Table 1. Parameters and actual values of coded levels used in the 2^{5-1} fractional factorial design experiments.

No.	Parameters	Coded	Type of parameters	Actual values of coded levels		Units
				-1	+1	
1	Fermentation time	A	Numerical	8	18	Days
2	Temperature	B	Numerical	30	50	°C
3	Addition of glucose	C	Numerical	0	50	g/L
4	Condition of waste	D	Categorical	Juice	Slurry	–
5	Part of waste	E	Categorical	Core	Peel	–

Analytical methods

pH and acid content

A pH meter (Mettler-Toledo AG, B211773648, 8603 Schwerzenbach) was used for all pH value measurements which were carried out at the end of the fermentation. The total acidity was estimated using 1.0 mL biovinegar sample, phenolphthalein and a neutralizing agent of 0.1 M NaOH, which yielded total acid content in percentage. This method was adopted from Raji et al. (2012).

Acetic acid concentration

The acetic acid concentration was quantified using HPLC (Agilent technologies, model number 7111B, serial number DEAET00386) adopted from Zhang et al. (2017) with modification. Samples were filtrated with a 0.45 µm membrane filter. A Synergy Hydro C18 250 organic acids column (300 × 4.6 mm, Japan) with sulphuric acid as mobile phase at 0.5 mL/min was used, measured with a UV detector at 221 nm (1260 VWD, 1200 series; Agilent Technologies).

Reducing sugar concentration

The reducing sugar was estimated using dinitrosalicylic acid (DNS) method of Teixeira et al. (2012). 1.5 mL of biovinegar sample with dilution of citrate buffer was added into 3 mL DNS reagent and the mixtures was heated at 100°C

for 5 min. After cooling to room temperatures, 2 mL of the mixture was withdrawn. Each sample was scanned with UV/VIS spectrophotometer (Model Genesys 50, serial number of 9A3WO53007) at wavelength 540 nm to obtain the optical density (OD) values and compared with the glucose calibration curve. Glucose calibration curve was developed earlier at concentration range between 0 – 100 g/L using the same procedure as the sample.

Fermentation kinetic development

Product yield, $Y_{p/s}$ was calculated by dividing the product (concentration of acetic acid) over substrate (consumed reducing sugar concentration). The productivity was calculated by dividing acetic acid concentration with its respected fermentation time.

EXPERIMENTAL RESULTS AND DISCUSSION

Screening on parameters affecting biovinegar production

Screening of parameters affecting the biovinegar production was carried out using 2^{5-1} fractional design to determine the degree of the effect to the response. Table 2 shows total acids concentration obtained from natural fermentation was between 0.93 to 1.22 % w/v.

Table 2. The result of the 2^6 fractional factorial experiments.

Run	Parameters					Acid concentration (% w/v)
	A: Time	B: Temperature	C: Addition of glucose	D: Condition of waste	E: Part of waste	
	Days	°C	%	–	–	
1	18	50.00	5.00	Core	Slurry	1.04
2	18	50.00	0.00	Peel	Slurry	0.84
3	18	30.00	5.00	Peel	Slurry	1.19
4	18	50.00	0.00	Core	Juice	0.93
5	8	50.00	5.00	Core	Juice	1.06
6	8	30.00	5.00	Peel	Juice	1.17
7	18	30.00	5.00	Core	Juice	1.13
8	8	50.00	0.00	Peel	Juice	1.05
9	8	30.00	5.00	Core	Slurry	1.08
10	**18**	**50.00**	**5.00**	**Peel**	**Juice**	**1.22**
11	8	30.00	0.00	Core	Juice	1.14
12	8	50.00	5.00	Peel	Slurry	0.99
13	18	30.00	0.00	Core	Slurry	1.04
14	8	30.00	0.00	Peel	Slurry	1.32
15	18	30.00	0.00	Peel	Juice	1.05
16	8	50.00	0.00	Core	Slurry	1.04

Main effect and its interaction on biovinegar production

The analysis of variance (ANOVA) was done to determine the statistical significance of the model suggested by the software. The significance of the model can be determined using *F*-values, while the *p*-values were used to examine the significance of each coefficient as shown in Table 3. From the model, *F*-value was 24.95 indicated that only 1.13 % chances that the model's *F*-value of this large could occur due to noise. Low *p*-value ($p < 0.0001$) showing the significance of the corresponding parameter (Masoumi et al., 2011). The model term effects *A*, *B*, *C*, *E*, *AC*, *AD*, *BC* and *CD* were statistically significant in affecting the acid production.

The satisfactory R^2 value of 0.9901 indicates best model fits the experimental values and predicted well. The final equations in term of coded parameters is shown in Equation (1):

$$Y = 0.18 - (9.844\times10^{-3})A - (4.531\times10^{-3})B + (4.719\times10^{-3})C - (2.219\times10^{-3})D - (3.906\times10^{-3})E + (2.301\times10^{-3})AB + (4.406\times10^{-3})AC - (5.031\times10^{-3})AD + (3.281\times10^{-3})AE + (9.969\times10^{-3})BC - (2.469\times10^{-3})BD - (3.594\times10^{-3})CD \quad (1)$$

Where *Y* is the response of acid yield, *A* is temperature, *B* is fermentation time, *C* is addition of glucose, *D* is condition of waste and *E* is part of waste. Parameters of *A*, *B*, *C*, *D* and *E* are referred as the main effect, while *AB*, *AC*, *AD*, *AE*, *BC*, *BD* and *CD* are the interaction effects. From the equation, main parameters *A*, *B*, *D* and *E* were negatively affected the fermentation. Meanwhile, parameter *C* affected the fermentation positively. Then, the interaction of parameters: *AB*, *AC*, *AE* and *BC* positively affected while *AD*, *BD* and *CD* negatively affected the acid production. Positive effect means, as the parameter increased, the acid production also increased, while the negative effect was vice versa.

Table 3. Test of significance for regression coefficient.

Source	Sum of squares	df	Mean of squares	*F*-value	*p*-value	
Model	5.406×10-3	12	4.505×10^{-4}	24.95	0.0113	significant
A-Temperature	1.550×10-3	1	1.550×10^{-3}	85.86	0.0027	
B-Fermentation time	3.25×10^{-4}	1	3.285×10^{-4}	18.19	0.0236	
C-Addition of glucose	3.563×10^{-4}	1	3.563×10^{-4}	19.73	0.0212	
D-Condition of waste	7.877×10^{-5}	1	7.877×10^{-5}	4.36	0.1280	
E-Part of waste	2.441×10^{-4}	1	2.441×10^{-4}	13.52	0.0348	
AC	3.106×10^{-4}	1	3.106×10^{-4}	17.20	0.0255	
AD	4.050×10^{-4}	1	4.050×10^{-4}	22.43	0.0178	
BC	1.590×10^{-3}	1	1.590×10^{-3}	88.05	0.0026	
CD	2.066×10^{-4}	1	2.066×10^{-4}	11.44	0.0430	
Residual	1.806×10^{-5}	3	1.806×10^{-5}			

Table 4 shows the contribution of main parameter and its interaction to acid production. The highest contributor was parameter *A* with 28.39 % contribution. Previous study showed an optimum temperature range for growth of *Acetobacter* and yeast which commonly used in biovinegar production was between 30 and 32 °C (Chakraborty et al., 2017b; Ghosh et al., 2014; Kong et al., 2018). Meanwhile, special thermo-tolerant *Acetobacter* was able to grow up to 40 °C (Arroyo-López et al., 2009). Thus, optimum temperature in biovinegar production will depend on the microorganism present either in the raw material or added as a starter. Wider temperature range was usually tested in factorial screening analysis. Thus, in this study, the temperature range was investigated between 30 and 50 °C.

The second highest contributing parameter was parameter C, with 6.52 %. A study by Raji et al. (2012) reported that the optimum level of glucose concentration was 2.5 % which is lower than the current study. Another parameter that was also considered significant was parameter B with the percentage of contribution of 6.02 %. Liu et al. (2019) took five days for alcoholic fermentation then additional five days for acetous fermentation to produce apple vinegar of 0.9 % acid content using mixed strains.

Table 4. Percentage of contribution of main parameter and their interaction.

Parameter	Contribution (%)
A-Temperature	28.39
B-Fermentation time	6.02
C-Addition of glucose	6.52
D-Condition of waste	1.44
E-Part of waste	4.47
AC	5.69
BC	29.12

Effect of main parameters

The effects of two independent parameters on the acid production are shown in Figure 1. Acid production decreased with increased temperature from 30 to 50 °C, as shown in Figure 1a. Although an optimum temperature range for *Acetobacter* and yeast was between 30 and 32 °C, but for special thermotolerant microorganism, it was able to grow at temperature up to 40 °C (Arroyo-López et al., 2009; Chakraborty et al., 2017b; Ghosh et al., 2014; Kong et al., 2018). Taking this into account, the temperature had to be kept in a wider range for screening study which was between 30 and 50 °C. Acid production was evidently increased as higher initial glucose concentration exist in the substrate as shown in Figure 1b. Glucose was used by the microorganism as the carbon source for both biosynthesis and energy production to support the microorganism growth and product formation.

Figure 1c showed that acid production was slightly decreased over fermentation time from 8th day to 18th day. Similar result was reported by Kong et al. (2018) where acetic acid content was increased at the beginning of fermentation and eventually decreased by 2.34 % as the fermentation continued until 12th day. Acetic acid evaporates easily, thus influencing the loss of acetic acid through evaporation when exposed to air, which might be the reason in acid decreased over time (Sanarico et al., 2003). Figure 1d showed that acid production was slightly decreased by 0.01 % w/v when the substrate was changed from peel waste to core waste. It can be concluded that there was no significant difference of acid production from both parts of waste. Study by Kodagoda and Marapana (2017) stated that pineapple peel contains higher lignocellulose than the core. Lignocellulose mainly composed of three groups of polymers, namely celulose, hemicellulose and lignin. Cellulose and hemicellulose are sugar rich fractions of interest for use in fermentation process, since microorganisms may use the sugars for growth (Mussatto & Teixeira, 2010). Therefore, maybe core do not have enough sugar to support the microorganism growth and acid production. Whereas, the peel has additional carbon sources from the lignocellulosic related to results shown in Figure 1d. This would be aiding in acid production that matches with the result of this study where higher acid was produced from the peel than the core substrate.

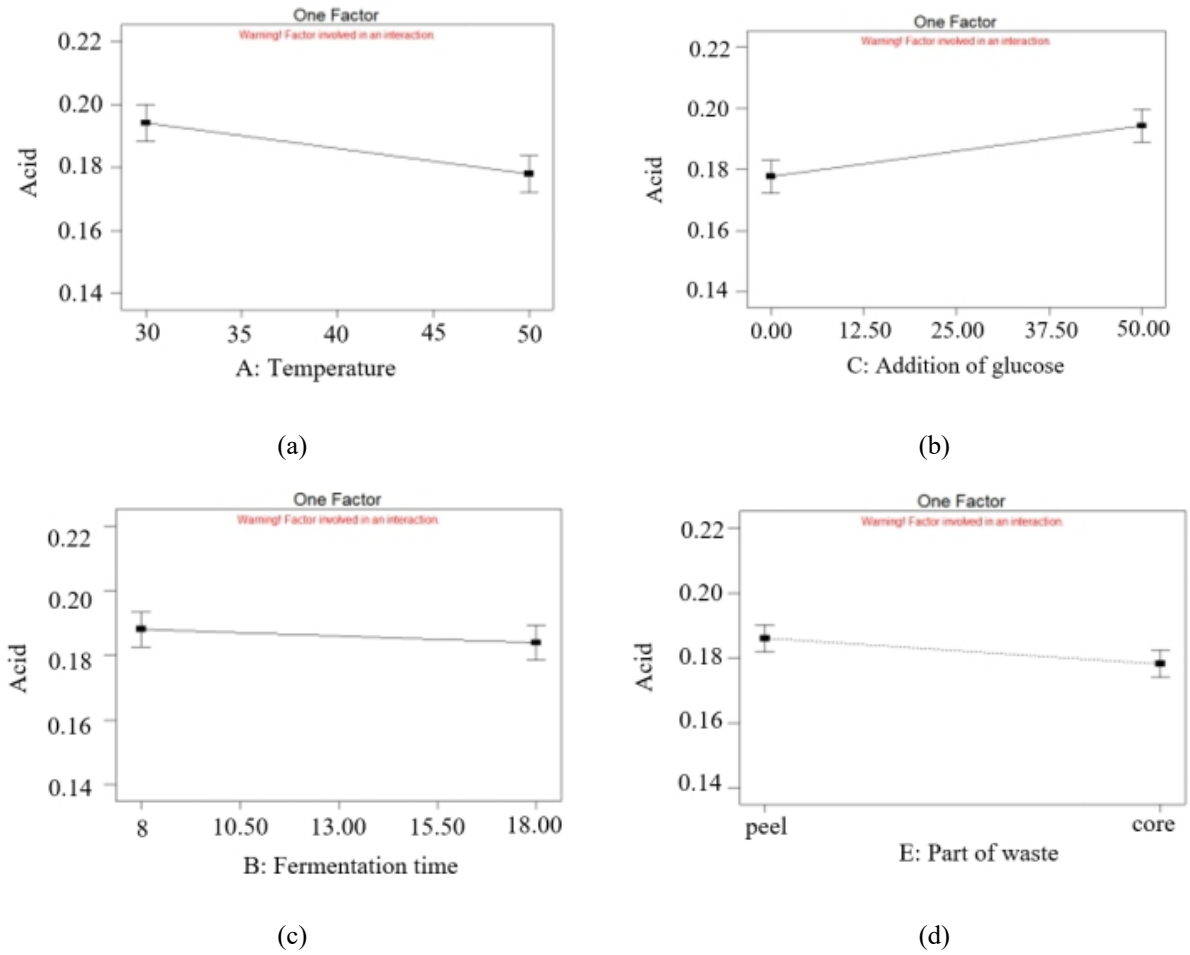

Figure 1. Effect of main parameters to acid production. (a) temperature; (b) addition of glucose; (c) fermentation time; (d) part of waste

Effect of parameter interaction

Figure 2 shows selected parameter interaction which has significant contribution towards acid production. At low temperature (30 °C) in both substrates (with and without glucose addition) higher acid was produced than at high temperature (50 °C) as shown in Figure 2a. Substrate with 50 g/L glucose addition produced a maximum of 0.2 % w/v final acidity in which it was 0.025 % w/v higher than that without glucose addition. It is apparent that fermentation maintained at an optimum temperature will thrive as reported by Sossou et al. (2009) and Roda et al. (2017). Temperature has to be kept in a wider range for screening study which between 30 and 50 °C considering the variety of mixed strain might exist in the substrate (Arroyo-López et al., 2009; Chakraborty et al., 2017b; Ghosh et al., 2014; Kong et al., 2018).

At short fermentation time (eight days) in both substrates (with and without glucose addition) produced acid at 0.19 % w/v as shown in Figure 2b. But when fermentation time increased to 18 days, substrate with additional glucose content produced higher acid production by 0.035 % w/v than that without glucose addition. Enough carbon source throughout the fermentation was important to sustain microorganism growth and product formation at extended time (Vijayakumar et al., 2015).

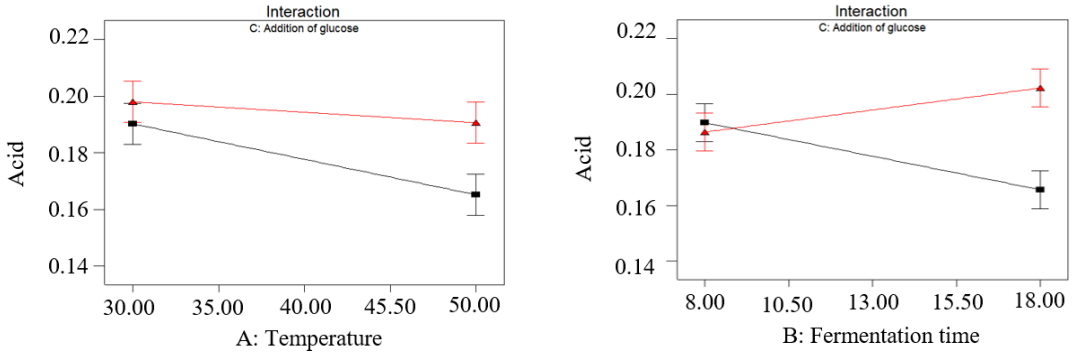

Figure 2. Effects of parameters interaction on acid production. (a) temperature and addition of glucose; (b) fermentation time and addition of glucose. Red line: 50 g/L glucose addition; black line: no glucose addition.

Validation of model

The model suggested by DoE® was validated experimentally through triplicate runs. The best fit condition suggested by the software was carried out at 30 °C, with 50 g/L glucose addition, for eight days of natural fermentation, using juice from the peel. The desirability of these condition was 0.867. The acid production was recorded at 1.12 % w/v with error of 2.91 % than the predicted production. The error was below 30 %, thus it is an acceptable error for biological experiment (Sharif et al., 2017). The fermentation kinetic developed in biovinegar production is shown in Table 5.

Table 5. Fermentation kinetics of biovinegar production.

Parameter	Value
Fermentation time (days)	8
Concentration of acid (% w/v)	1.12
Predicted concentration of acid (% w/v)	1.15
Concentration of acetic acid (% w/v)	0.94
Initial reducing sugar available (% w/v)	5.81
Final reducing sugar available (% w/v)	0.28
Reducing sugar consumption (%)	95.04
Initial pH	4.00
Final pH	3.57
Yield of acetic acid, $Y_{p/s}$ (g/g)	0.1699
Productivity of acetic acid (g/L.h)	0.0489
Validation error (%)	2.91

CONCLUSION

Factorial screening of biovinegar utilizing pineapple waste by natural fermentation was successfully conducted. Fractional factorial screening was able to determine the effect of the five parameters on acid production. Fermentation temperature contributed the most to the production of acid as much as 28.39 %. This was followed by the addition of glucose, fermentation time and part of waste with contribution of 6.52 %, 6.02 % and 4.47 %, respectively. Based on the ANOVA, the model was statistically significant with R^2 of 0.9901. Two main parameter interactions were identified to be significant; between temperature (A) and addition of glucose (C), also fermentation time (B) and addition of glucose (C). Validation run proved that the model and suggested condition by the software was reliable, producing biovinegar with 1.12 % w/v acidity with an error of 2.91 %. The results show that fractional factorial design is suitable to be used in the investigation of many parameters with a minimum number of experiments.

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