https://scilett-fsg.uitm.edu.my/
Vol. 16(1) JANUARY 2022

ISSN: 1675-7785
eISSN: 2682-8626

DOI: 10.24191/sl.v16i1.13651

Screening of Lactic Acid Fermentation by *Rhizopus oryzae* on Banana Peel Using Two-Level Factorial Analysis

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Received: 3 June 2021; Accepted: 27 July 2021; Published: 14 January 2022

ABSTRACT

For ages, pure sugars or edible crops have produced lactic acid. However, a major concern on lactic acid production lies in the cost of the raw materials used. Thus, an alternative to overcome this situation is urgently needed. Characterization of banana peels shows that it contains promising sugar that can be utilized for lactic acid production, which are xylose (0.774 g/L), glucose (0.756 g/L) and fructose (0.532 g/L). Thus, this study aims to screen the potential of banana peel as a substrate by using *Rhizopus oryzae* through batch fermentation for lactic acid production, as *R. oryzae* can synthesize lactic acid in low nutrient requirements. Two-level factorial analysis was designed to screen the effects of moisture content (60% and 80%), temperature (27 °C and 40 °C), pH (4.5 and 6.5) and inoculum size ($1x10^4$ spores/mL and $1x10^8$ spores/g) on the lactic acid production. Based on the Two-level factorial (2LF) analysis, the highest lactic acid production of 0.0813 g/L was detected at 80 % moisture content, pH 4.5, the temperature of 27 °C and inoculum size of 1x104 spores/g. The findings show that most of the conditions have a significant difference between each other ($p<0.05$). Therefore, the fermentation of banana peels by *R. oryzae* could be a promising method to produce a lactic acid concentration.

Keywords: *Banana peel, batch fermentation, lactic acid, Rhizopus oryzae, two-level factorial*

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INTRODUCTION

Lactic acid is a natural organic acid widely used in food preservatives and flavours. It is also useful in non-food industries such as cosmetics, pharmaceutical, chemical industries, and textiles. About 85 % of lactic acid has been utilised as a part of sustenance and nourishment related applications in the USA and generate biodegradable and biocompatible polylactate polymers [1]. According to the Global Lactic Acid Market [2], by 2022, the lactic acid value is projected to reach USD 4,129.19 million, growing at a CAGR of 16.9 % from 2015 to 2022 [2]. Furthermore, the global market for lactic acid in 2022 is expected to reach 1,844.56 kilo tonnes compared to 2014 with 750.00-kilo tonnes. Chemical synthesis typically forms a mixture of L-lactic acid and D-lactic acid, while microbial fermentation produces pure L-lactic acid or D-lactic acid [3].

However, in food and drug industries, L-lactic acid is preferred as D-lactic acid could harm the human's body. Industries have turned to the prevailing strategy of producing lactic acid by microbial fermentation utilising suitable microorganisms due to ecological concerns, low production temperature, low energy necessities, and high purity [4]. Thus, food waste could be a potential material for lactic acid fermentation due to its high starch content and the convenience of obtaining it as it is available in large quantities [5]. In industries, a substrate such as raw glucose synthesises lactic acid. However, it is costly, and therefore, substrate replacement is urgently needed. Thus, lignocellulosic biomass from crops residues was considered to overcome this situation. Lignocellulosic biomass is a cost-effective, abundant, and renewable source that can be used to produce products such as biofuels, biomolecules, biomaterials and bioenergy [6]. Agricultural waste such as banana peel rich in lignocellulose, sucrose, glucose, and fructose is believed to be a great alternative as a substitute for glucose in industrial lactic acid production [7].

In Malaysia, banana ranches cover up to 26,000 hectares with an aggregate generation of 530,000 metric tons each year, making it the second most broadly developed organic product [8]. The abundance of banana peel sources could reduce the operational cost in industries and help manage agricultural waste simultaneously. Instead of Lactic Acid Bacteria (LAB), *Rhizopus oryzae* is preferred to synthesise lactic acid from agricultural waste [1]. *R. oryzae* is preferable because it does not require the purification process as it can directly produce pure L (+)-lactic acid [9]. However, limited research is conducted on optimising factors that influence lactic acid production using banana peel as a substrate through batch fermentation by *R. oryzae*. Most fermentation methods are limited to certain techniques and the same strain [10]. Thus, in this research, the screening of lactic acid based on identified parameters (moisture content, temperature, pH and inoculum size) was conducted using Two-level factorial analysis via Statistical Package for the Social Sciences (SPSS) software [11].

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EXPERIMENTAL

Cultivation of R. oryzae

R. oryzae was obtained from Universiti Teknologi Malaysia, Johor. The fungi were inoculated on Potato Dextrose Agar (PDA) using inoculum loops and incubated at 37 °C for seven days. The spores were harvested and suspended in 1 % Tween 80 before centrifuging at 4000 rpm for 15 minutes. The spores were then calculated using a haemocytometer and observed before being transferred into the fermentation's flask [12].

Preparation of Feedstock

This lab-scale experiment was prepared by drying 400 g of banana peel collected from a food stall around Arau, Perlis. The samples were then dried in an oven at 50 °C for three days before being ground to fine particles. Then, the banana peel powder was autoclaved at 121°C for 15 minutes in an Erlenmeyer flask capped with a cotton stopper [13]. The sugar content (xylose, glucose and fructose) in banana peel was screened by mixing 1 g of samples with 0.01M H_2SO_4 for 5 minutes before centrifuging for 10 minutes at 4000 pm. The supernatant was detected using an HPLC-UV spectrophotometer. The operating conditions were set up as follows: flow rate at 0.6 mL/min, a mobile phase of acetonitrile: water (8:2), run time for 10 minutes and wavelength at 195 nm [14].

Medium preparation

The enriched broth medium for the fermentation was prepared as follows: 2.0 g/L $(NH_4)_2SO_4$, 0.25 g/L $MgSO_4$. $7H_2O$, 0.04 g/L $ZnSO_4.7H_2O$, 0.2 g/L KH_2PO_4, 10 g/L $CaCO_3$, 0.03 g/L $MnSO_4.H_2O$, and 0.03 g/L $FeSO_4.H_2O$ were weighed and mixed with 1 L distilled water [15].

Batch fermentation

The batch fermentation was conducted by adding 2 g of banana peel powder into the Erlenmeyer flask containing 50 mL of the enriched broth medium as prepared above [16]. The *R. oryzae* was fed with 2 g of banana peel powder daily for five days, and 1 ml of the sample was aliquoted every day and centrifuged for 5 minutes at 4000 rpm to get the supernatant [16]. The supernatant was then filtered using 0.2 µm Millipore membrane filters to the vial and analyzed using an HPLC-UV spectrophotometer. The flask containing 50 mL of distilled water and banana peel powder was set up as a control.

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Experimental Design

It is necessary to consider the specificity of lactic acid produced by specific microorganism strains. This is due to the different strains that influenced the rate of lactic acid production [10]. The factors influencing the lactic acid production have been screened using a Two-level factorial design via SPSS Software Version 20, and α was set to 0.05 [17], and at a high and low level in each independent variable [18]. Table 1 shows the range of factors influencing lactic acid production using a Two-level factorial design

Table 1: Range of factors influencing the lactic acid production using two-level factorial design

Factors	Low Level (-)	Higher Level (+)
pH	4.5	6.5
Temperature (°C)	27	40
Moisture content (%)	60	80
Inoculum size (spores/mL)	$1x\ 10^4$	$1x10^8$

RESULTS AND DISCUSSION

Banana Peel as a Substrate for Rhizopus oryzae

Based on the previous statement, banana peel is a suitable substrate for lactic acid production since it contains sugars such as xylose, glucose and fructose. Table 2 shows the sugar content in the banana peel.

Table 2: Sugar content in banana peel

Type of Sugar	Sugar content (g/L)	Percentage of sugar contents (%)
Xylose	0.774	37.54
Glucose	0.756	36.67
Fructose	0.532	25.80

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It was found that the highest sugar content in banana peel is xylose (0.774 g/L), followed by glucose (0.756 g/L) and fructose (0.532 g/L). The presence of the monosaccharides in banana peel is the precursor to the lactic acid production by *R. oryae* [19]. In addition, starchy materials, and pentose sugar, such as xylose, is expected to produce pure lactic acid [20]. Compared to other types of sugar, glucose and xylose are vital and feasible because they can efficiently be converted into target material [21].

Factors' Influence on Production of Lactic Acid

Sixteen conditions were set up for the lactic acid analysis using Two-level factorial as Table 3. The inoculum size, moisture content, pH, and temperature were varied for the optimization, and the response of lactic acid condition in five days was recorded.

Table 3: Lactic acid production based on two-level level factorial for the screening

Condition	Inoculum size (spores/mL)	Moisture Content (%)	pH	Temperature (°C)	Average of lactic acid produced in 5 days (g/L)
A	1×10^4	60	4.5	27	0.0133
B	1×10^4	60	6.5	27	0.0301
C	1×10^4	80	4.5	27	0.0373
D	1×10^4	80	6.5	27	0.0813
E	1×10^4	60	4.5	40	0.0065
F	1×10^4	60	6.5	40	0.0391
G	1×10^4	80	4.5	40	0.0591
H	1×10^4	80	6.5	40	0.0744
I	1×10^8	60	4.5	27	0.0397
J	1×10^8	60	6.5	27	0.0583
K	1×10^8	80	4.5	27	0.0076
L	1×10^8	80	6.5	27	0.0673
M	1×10^8	60	4.5	40	0.0238
N	1×10^8	60	6.5	40	0.0071
O	1×10^8	80	4.5	40	0.0343
P	1×10^8	80	6.5	40	0.0058

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At 80 % moisture content, the *R. oryzae* could utilize sugar efficiently and lead to the optimum production of lactic acid. However, increasing the moisture content would not increase lactic acid concentration beyond this point. This is probably because the fungus has entered the stationary phase, where product accumulation slows lactic acid synthesis [12]. This will prevent the activation of alternative metabolism pathways and the production of undesirable by-products such as ethanol, fumaric acid, and CO_2 [13]. The moisture content is also related to the temperature because high temperature will disrupt the fungal physiology and acid metabolism, which leads to low secretion of lactic acid [12]. This explained why condition P could only secrete a low amount of lactic acid even with high moisture content. The high temperature (40 °C) affected lactic acid production as the fungi need to adapt to the high temperature [22].

It was also found that the optimum pH for the lactic acid secretion is around pH 5-6.5, and the amount of lactic acid could decline if the pH is decreased [23]. This is because pH plays a crucial role in regulating different metabolic functions, reflecting the amount of lactic acid produced [24,25]. Meanwhile, the high number of inoculum (1 X 10^8 spores/mL) in P led to decreasing lactic acid production because the fungi had to compete for the substrate. The same pattern was observed when solid pineapple waste was fermented by *R.oryzae* [12].

Lactic Acid Production for Five Days of Fermentation

The lactic acid production between 16 conditions set up was recorded and compared. Figure 1 represents the lactic acid production (g/L) by *R. oryzae* for five days of incubation. Based on Figure 1, the highest lactic acid production was detected on Day 3 in most of the runs. Previous researchers also observed similar patterns, which was attributed to the formation of mycelia that gradually increased within 72 hours [25,26].

The formation of mycelia decreased after 84 hours, and this affects the production of lactic acid, as the log phase of fungi is between 12 to 72 hours (long log phase) [20]. The optimum temperature for the lactic acid produced by *R. oryzae* is 27 °C. Treatment N and treatment P had a high temperature which was 40 °C. High temperature can cause fungal physiology to change and disrupt the metabolism of lactic acid, which reduces lactic acid production [12].

As for pH for treatment N and P, the value is higher, not suitable for lactic acid production. The higher the pH for *R. oryzae,* the more likely it will produce lactate instead of lactic acid. The optimum pH for lactic acid produced by *R. oryzae* is between pH 5 to 6.5. In addition, a high amount of fungal spore leads to substrate competition. This will cause lactic acid production to be reduced as in treatment K, N, and P [12].

https://scilett-fsg.uitm.edu.my/
Vol. 16(1) JANUARY 2022

ISSN: 1675-7785
eISSN: 2682-8626
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DOI: 10.24191/sl.v16i1.13651

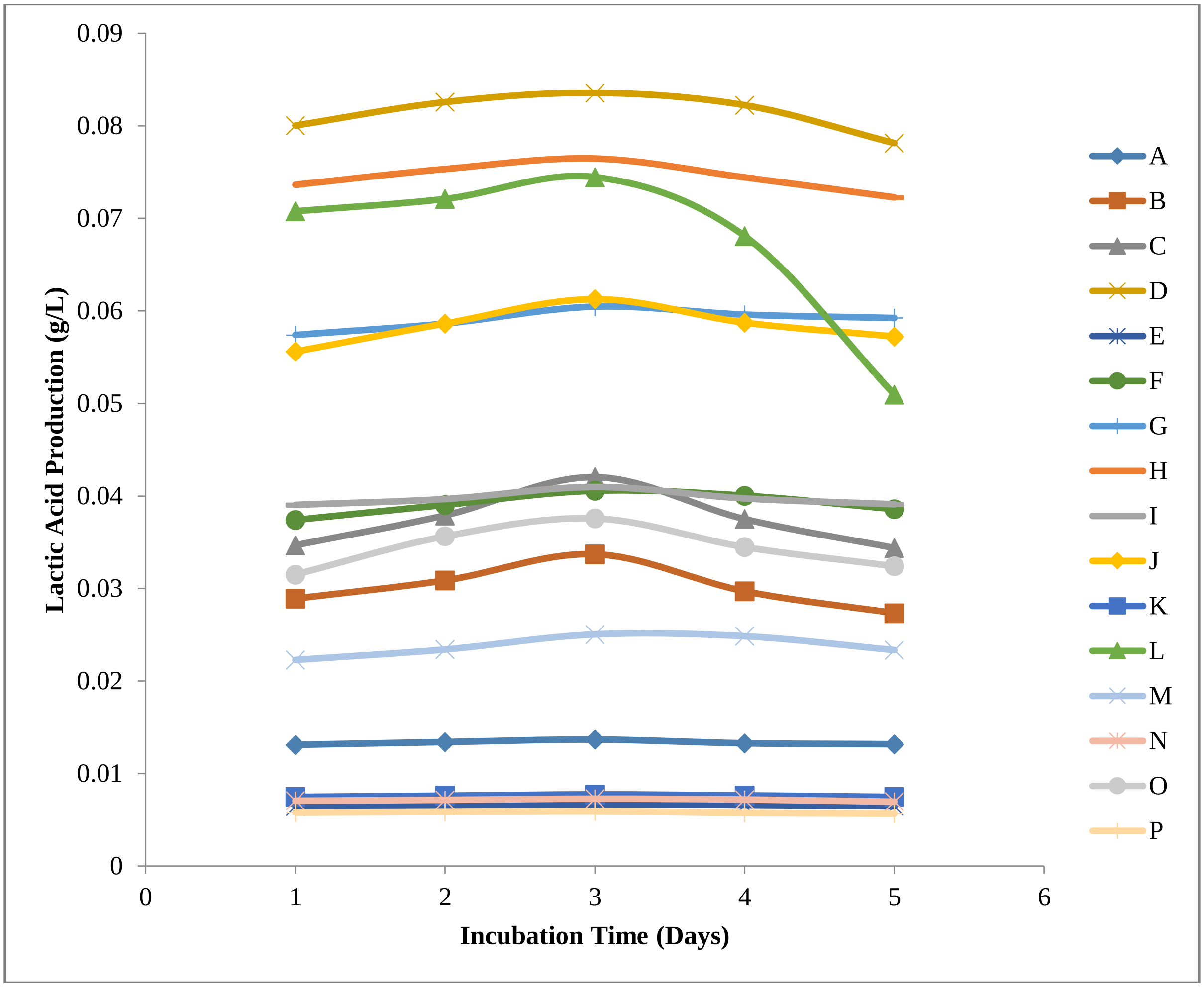

Figure 1: Average of Lactic Acid for Five Days of Fermentation

ANOVA Analysis

Analysis of Variance (ANOVA) for lactic acid production was conducted using SPSS software. This is to determine the level of significance of the main effect for each of the factors and their interaction. Table 4 shows the significant differences in lactic acid production. Two-level factorial analysis was conducted to compare the effect of lactic acid production on two-level optimization conditions, including moisture content (%), pH, temperature (°C) and inoculum sizes (spores/gram). There was a significant effect on lactic acid production when different environmental conditions were set as the $p<0.05$ with $F(15, 64)=408.627$, p.000. As shown in

ISSN: 1675-7785
eISSN: 2682-8626

DOI: 10.24191/sl.v16i1.13651

Figure 1, factors K, N and P show almost a flat line, indicating no significant difference. Thus, lactic acid production by these three factors is almost the same.

Table 4: Analysis of Variance (ANOVA) for Lactic Acid Production

	Sum if squares	**Degree of Freedom**	**Mean Square**	**F- value**	**Significance**	**R^2 value**
Between group	0.048	15	0.003	408.627	0.000	0.1792
Within group	0.001	64	0.000			
TOTAL	0.0049	79				

CONCLUSION

This lab-scale study has significantly shown that banana peel could be utilised as a substrate to produce lactic acid through fed-batch fermentation using *R. oryzae*. The banana peel contained 0.774 g/L, 0.756 g/L and 0.532 g/L of xylose, glucose and fructose. The highest production of lactic acid of 0.0813 g/L was identified at the suggested condition by two-level factorial analysis where the condition was set up at $1x10^4$ spores/g of inoculum size, 80% moisture content, pH 6.5 and temperature of 27 °C. This research successfully shows the potential of banana peel to be used as an alternative to producing lactic acid in future.

ACKNOWLEDGMENTS

The authors would like to thank Universiti Teknologi MARA, Perlis for providing laboratory space and equipment in carrying out this study and Universiti Teknologi Malaysia for the microbial culture.

AUTHOR’S CONTRIBUTION

Nurul Syafiqah and Atikah Kamaluddin had carried out the research and wrote the article. Nor Atikah Husna had conceptualised the central research idea and provided the theoretical framework. Nor Atikah Husna, Nor Azimah Mohd Zain, Sharir Aizat and Siti Nur Balqis had designed the research, supervised research progress and revised the article.

ISSN: 1675-7785
eISSN: 2682-8626

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CONFLICT OF INTEREST STATEMENT

The authors agree that this research was conducted in the absence of any self-benefits, commercial or financial conflicts and declare absence of conflicting interests with the funders.

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Vol. 16(1) JANUARY 2022

ISSN: 1675-7785
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