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DETERMINATION OF LACTIC ACID PRODUCTION BY RHIZOPUS ORYZAE IN SOLID STATE FERMENTATION OF PINEAPPLE WASTE

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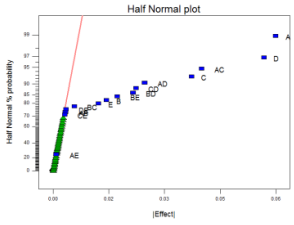
Graphical abstract



Abstract

Solid pineapple waste (SPW) is one of the most abundant agricultural wastes found in tropic region. This study is looking into the potential of utilising solid pineapple waste in solid state fermentation for the production of lactic acid by *Rhizopus oryzae*. A 2-level factorial design was employed to screen the effect of moisture content (60% and 80%), inoculum size (1×10^4 spores/g and 1×10^8 spores/g), pH (4.5 and 6.5), temperature (27°C and 40°C) and particle size (<0.5 mm and >3.15 mm) to the production of lactic acid. The predicted maximum production is 0.0221 g lactic acid/g SPW in SSF condition of 80% moisture; pH 6.5; 1×10^4 spores/g of inoculum; waste particle of 3.15 mm; and temperature 27°C. Analysis of variance (ANOVA) showed that the model is significant with high value of predicted (0.9616) and adjusted (0.9726) R-squared, indicated a good agreement between the predicted and actual values at each point of the experiment. Post-statistical experiment confirmed the ability of lactic acid production by *R. oryzae* at the predicted conditions with 0.0236 g lactic acid/g SPW being produced.

Keywords: Solid pineapple waste (SPW), lactic acid, solid-state fermentation, 2-level factorial, *Rhizopus oryzae*

Abstrak

Sisa pepejal nanas merupakan salah satu daripada bahan buangan pertanian yang paling banyak boleh didapati di kawasan tropika. Kajian ini bertujuan bagi melihat keboleh-upayaan penggunaan sisa pejal nanas bagi fermentasi dalam keadaan pepejal untuk menghasilkan asid laktik oleh *Rhizopus oryzae*. 2-tingkat reka bentuk faktorial telah digunakan bagi melihat kesan kelembapan (60% dan 80%), saiz inokulum (1×10^4 spora/g dan 1×10^8 spora/g), pH (4.5 dan 6.5), suhu (27°C dan 40°C) dan saiz sampel (<0.5 mm dan >3.15 mm) terhadap pengeluaran asid laktik. Diramalkan pengeluaran maksimum asid laktik sebanyak 0.0221 g asid laktik/g SPW dengan menggunakan 80% kelembapan: pH 6.5: inokulum sebanyak 1×10^4 spora/g; sampel bersaiz 3.15 mm; dan suhu 27°C. Analisis varians (ANOVA) menunjukkan bahawa model ini adalah signifikan dengan nilai yang diramalkan (0.9616) dan nilai ubah suai (0,9726) R-kuasa dua, di mana ia menunjukkan hubungan yang baik di antara nilai yang diramalkan dan nilai sebenar pada setiap titik eksperimen. Eksperimen pasca-statistik mengesahkan bahawa keboleh-upayaan pengeluaran asid laktik oleh *R. oryzae* pada keadaan yang telah diramalkan ialah sebanyak 0.0236 g asid laktik/g SPW.

Kata kunci: Sisa pepejal nanas, asid laktik, fermentasi keadaan pepejal, 2-tingkat reka bentuk factorial, *Rhizopus oryzae*

1.0 INTRODUCTION

Pineapple (*Ananas comosus*) is a kind of tropical plant which is believed to originate from eastern South America [1]. It was introduced in Malaysia in the 16th century during the era of rubber crop development in 1921 where it began to be planted as commodity in Singapore, Johor and Selangor. Pineapple is best grown in peat soil area, mostly occurred in the state of Johor and results in extensive growth pineapple processing plants. Unfortunately, the solid pineapple wastes from food processing known as by-product resulting from the extraction of pineapple juice processes may lead to the accumulation of agriculture waste and eventually causing an environmental pollution [2]. Besides of the negative impact, it can be used for the production of value added products since the residual pulp, peels or skin of solid pineapple waste contains high amount of carbon sources [3]. High sugar content in the solid pineapple waste made it economically feasible for organic acid production.

Recently, fermentation process has become more industrially successful because of the increasing demand for naturally produced lactic acid [4]. Lactic acid production by fungi, such as *Rhizopus oryzae* has gained much attention. It also has advantages compared to bacteria, including their amylolytic characteristics, low nutrients requirement and valuable fermentation by-product [5]. Besides, fungi were listed as the most suitable microorganism for solid state fermentation (SSF) due to their capability to penetrate and absorb the nutrients from solid waste [6].

Solid substrate can be described as nutrition carrier substrate or inert carrier substrate [7]. The use of solid sample as a bed material that is impregnated with nutrients could enhance lactic acid production. Ghosh and colleague (2011) reported 44.88 g/L of lactic acid production using pine needles soaked in 120 g/L of pure whey as substrate,with co-cultured *Lactobacillus delbrueckii* (NCIM2025) and *Lactobacillus pentosus* (NCIM 2734) [8]. This study reports the potential use of nutritional substrate of solid pineapple waste (SPW) for lactic acid production by *Rhizopus oryzae* via 2-level factorial design. Findings from this study could be used as preliminary data for further improvement lactic acid production.

2.0 EXPERIMENTAL

Solid pineapple waste was heat-dried in a 60°C incubator for 7 days [9] and was grinded to fine particles using a grinder (WARING, USA). The sample was then sieved in order to separate the waste particles using 0.5, 1.0, 2.0, 3.15 mm of Endecotts test sieves (UK).

Rhizopus oryzae was maintained and the spores were germinated on Potato Dextrose Agar (PDA) for seven days of incubation at 37°C. For the purpose of sterilization, 10 g of dried grinded solid pineapple waste was autoclaved at 121°C for 15 minutes in a 250 mL Erlenmeyer flasks capped with cotton stopper.Next, the fungus was transferred onto fermentation medium containing 2.0 g/L $(NH_4)^2SO_4$, 0.25 g/L $MgSO_4 \bullet 7H_2O$, 0.04 g/L $ZnSO_4 \bullet 7H_2O$, 0.2 g/L KH_2PO_4 and 10 g/L $CaCO_3$.

Characterization of sugars contained in 1 g of SPW was conducted by mixing 1 g of sample with 0.01M H_2SO_4 (1:20). Then, the mixture was mixed for 5 minutes and centrifuged for 10 minutes at 4000 rpm. The supernatant was used to measure the reducing sugar by using the 3,5-dinitrosalicyclic acid (DNS) method [10]. However, the the content of glucose, fructose and xylose were detected (by High performance liquid chromatography (HPLC), using a 300 mm × 7.8 mm, Rezex RCM-Monosaccharide column (Phenomenex) with Refractive Index Detector (RID). The supernatant was then filtered through 0.2µm Milipore membrane filters into HPLC vials. The eluent used is 100% nanopure water at a flow rate of 0.6 mL/min and 10 minutes of post time.

Table 1 Variable of Real Values during Screening Using 2-Level Factorial Design

Variables	Component	Low level (-1)	High level (+1)
A	Moisture content, % (w/v)	60	80
B	Inoculum size (spores/g)	1×10^4	1×10^8
C	Temperature (°C)	27	40
D	pH	4.5	6.5
E	Particle size (mm)	<0.5	>3.15

The factors influencing lactic acid production were screened using 2-level factorial design. Five variables factors, which are moisture content, inoculum size, pH, temperature, and particle size, were expected to have a significant effect on lactic acid production (Table 1). The design contains a total of 48 experimental trials. Each independent variable was investigated at high (+1) and low (-1) level. The statistical analysis was used to identify the effect of each variable on lactic acid production. The experiments were randomized for statistical reasons.

In this experiment, 10 g of SPW has been used. During the screening study, 1 g of samples was withdrawn every24 hours and the extraction method was similar as the characterization method mentioned earlier. Then, the supernatant was used for the lactic acid, sugars (glucose, fructose and xylose) and reducing sugar concentration analysis. The lactic acid concentration in the samples was determined by using a HPLC. 0.005N H_2SO_4 was used as mobile phase. The stationary phase was Phenomenex with flow rate 0.5 mL/min and 80°C. The RID detector was used in this project with 60 min stop time and 10 min post time.

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3.0 RESULTS AND DISCUSSION

Solid pineapple waste that has been used consists of reducing sugar (1.429 g/g SPW), glucose (1.029 g/g SPW), fructose (0.764 g/g SPW) and xylose (0.183 g/g SPW). *R. oryzae* was used in this study since it is capable to perform a single stage SSF process to produce lactic acid. According to Karimi *et.al* (2006), *Rhizopus sp*. has a high enzymatic and metabolic capability that can use the polyoses as a carbon source for lactic acid production [11].

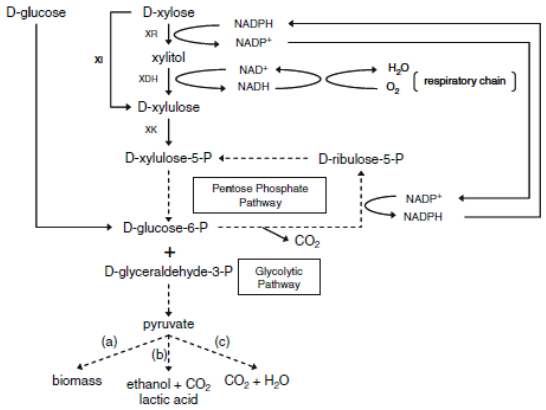

Figure 1 Possible pathway of lactic acid metabolism by *Rhizopus oryzae* [12]

R. oryzae utilizes sugars in aerobic condition. Insufficient aeration could reduce the efficiency of fungus to produce lactic acid and it might cause the activation of alternative metabolism pathway, at which finally yield the undesired by-products, such as ethanol, fumaric acid, CO_2, etc [12-14].

R. oryzae secretes the cellulolytic (endoglucanases, cellobiohydrolases, β-glucosidase) and xylanolytic (xylanase) enzymes to degradate the lignocellulosic component [14-17]. Metabolism of sugars includes glucose, fructose, and xylose occur in Embden-Meyerhof-Parnas pathway (EMP pathway) (Figure 1). Lactate dehydrogenase (LDH) on the other hand, plays a key role in driving lactic acid production.

As shown in Table 2, each independent factor was studied at a high and lower level leading to a total of 48 experiments. The maximum lactic acid concentration was detected at standard order 30, where 0.0234 g lactic acid/g SPW with 72.02%, 68.66% and 78.62% of glucose, fructose and xylose consumption, respectively. However the minimum production was detected at standard order 17, where 0.0025 g lactic acid/g SPW with 33.36%, 30.52% and 36.82% of glucose, fructose and xylose consumption, respectively. The amount of cellulose, hemicelluloses, extractives, and lignin found in raw SPW were 38.52%, 47.40%, 5.18%, and 8.9%, respectively. After the introduction of *Rhizopus oryzae* to SPW, the lignocellulosic components of SPW are fermented. Approximately about 8.16%, 19.44%, 2.53%, and 3.15% of cellulose, hemicelluloses, extractives, and lignin from SPW remained. From the results, it can be seen that *R. oryzae* utilize the sugars for lactic acid production efficiently. However, the production is not high possibly due to utilization of SPW as the sole nutrient for the *R. oryzae* and no addition of sugar hydrolysates during SSF.

Interpretation of results was analyzed using the analysis of variance (ANOVA) as appropriate to the experimental design used. Table 3 shows the ANOVA analysis for the suggested model of lactic acid concentration.

Table 2 Experimental design of screening factors for lactic acid production using 2-level factorial design

Run	Moisture content (%) *A*	Inoculum size (spores/g) *B*	Temperature (°C) *C*	pH *D*	Particle size (mm) *E*	Lactic acid concentration (g/g SPW)
1	60	1×10^4	27	4.5	>3.15	0.0161
2	60	1×10^4	27	4.5	>3.15	0.0152
3	60	1×10^4	27	4.5	>3.15	0.0124
4	80	1×10^4	27	4.5	<0.5	0.0083
5	80	1×10^4	27	4.5	<0.5	0.0078
6	80	1×10^4	27	4.5	<0.5	0.0090
7	60	1×10^8	27	4.5	<0.5	0.0155
8	60	1×10^8	27	4.5	<0.5	0.0154
9	60	1×10^8	27	4.5	<0.5	0.0142
10	80	1×10^8	27	4.5	>3.15	0.0092
11	80	1×10^8	27	4.5	>3.15	0.0066
12	80	1×10^8	27	4.5	>3.15	0.0093
13	60	1×10^4	40	4.5	<0.5	0.0144
14	60	1×10^4	40	4.5	<0.5	0.0159
15	60	1×10^4	40	4.5	<0.5	0.0170
16	80	1×10^4	40	4.5	>3.15	0.0036
17	80	1×10^4	40	4.5	>3.15	0.0025
18	80	1×10^4	40	4.5	>3.15	0.0030

Run	Moisture content (%) *A*	Inoculum size (spores/g) *B*	Temperature (°C) *C*	pH *D*	Particle size (mm) *E*	Lactic acid concentration (g/g SPW)
19	60	1×10^8	40	4.5	>3.15	0.0153
20	60	1×10^8	40	4.5	>3.15	0.0155
21	60	1×10^8	40	4.5	>3.15	0.0155
22	80	1×10^8	40	4.5	<0.5	0.0068
23	80	1×10^8	40	4.5	<0.5	0.0050
24	80	1×10^8	40	4.5	<0.5	0.0058
25	60	1×10^4	27	6.5	<0.5	0.0218
26	60	1×10^4	27	6.5	<0.5	0.0216
27	60	1×10^4	27	6.5	<0.5	0.0217
28	80	1×10^4	27	6.5	>3.15	0.0213
29	80	1×10^4	27	6.5	>3.15	0.0223
30	80	1×10^4	27	6.5	>3.15	0.0234
31	60	1×10^8	27	6.5	>3.15	0.0145
32	60	1×10^8	27	6.5	>3.15	0.0162
33	60	1×10^8	27	6.5	>3.15	0.0162
34	80	1×10^8	27	6.5	<0.5	0.0197
35	80	1×10^8	27	6.5	<0.5	0.0198
36	80	1×10^8	27	6.5	<0.5	0.0192
37	60	1×10^4	40	6.5	>3.15	0.0193
38	60	1×10^4	40	6.5	>3.15	0.0198
39	60	1×10^4	40	6.5	>3.15	0.0183
40	80	1×10^4	40	6.5	<0.5	0.0110
41	80	1×10^4	40	6.5	<0.5	0.0123
42	80	1×10^4	40	6.5	<0.5	0.0101
43	60	1×10^8	40	6.5	<0.5	0.0188
44	60	1×10^8	40	6.5	<0.5	0.0172
45	60	1×10^8	40	6.5	<0.5	0.0171
46	80	1×10^8	40	6.5	>3.15	0.0069
47	80	1×10^8	40	6.5	>3.15	0.0007
48	80	1×10^8	40	6.5	>3.15	0.0060

Table 3 Analysis of Variance (ANOVA) for the Production of Lactic Acid

Source	Sum of squares	Degree of freedom	Mean square	F-value	P > F	R^2
Model	0.16	12	0.013	140.09[a]	<0.0001[b]	0.9796
Residual	0.003260	35	0.00009314	-	0.3998[c]	
Lack of fit	0.0002827	3	0.00009424	1.01	-	
Pure error	0.002977	32	0.00009304	-	-	
Correlation total	0.16	47	-	-	-	

[a]F-value is significant. [b]model is significant, with P > F less than 0.005. [c]model is fit due to insignificant F-value. Standard deviation is 0.009651.

As mentioned previously, the associated probability denoted by "Prob > F" for factorial model is well below 0.05, which implies the factorial model is significant. The level of significance of the main effect for each of the factors as well as their interaction was also examined via ANOVA.

From the analysis, affecting factors identified were moisture content (factor A), inoculum size (factor B), temperature (factor C), pH (factor D), and particle size (factor E). However, the interacting factors were moisture content and temperature (factor AC), moisture content and pH (factor AD), inoculum size and temperature (factor BC), inoculum size and pH (factor BD), inoculum size and particle size (factor BE), and temperature and pH (factor CD) are significant model terms with Prob > F value is less than 0.05. Thus, all of these factors are significantly affect the lactic acid concentration in a factorial manner.

Significant factors that affected the response (lactic acid concentration) was analyse by the half normal plot graph in Figure 2. According to the plot, determination of ranks of the absolute value of various effects can be detected through this graph. The negligible factors is lie along the straight line, however the other factors and their cross-interaction give significant effect towards the response [18]. Moreover, the effect for moisture content (factor A) obviously falls far away from the line, where it shows an important signal.

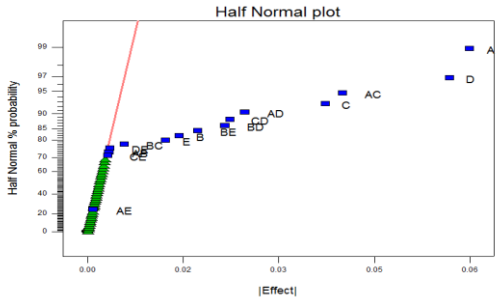

Figure 2 The Half Normal Plot

The "Pred R-Squared" of 0.9616 is in reasonable agreement with the "Adj R-Squared" of 0.9726. The adequate precision (Adeq. Precision) is essentially a tool that compares the range of the predicted values at the design points to the average prediction error. A value of adequate precision greater than 4 is desirable as it signifies sufficient model discrimination. The empirical model expressed in terms of coded variables for lactic acid concentration is represented by Equation 4.1.

Lactic acid concentration g/g SPW =

+ 0.14 -0.031 *A -7.479E-003 *B – 0.019 *C + 0.030 *D – 6.354E-003 *E + 1.729E-003 *A*B – 0.021 *A*C + 0.031 *A*D + 4.375E-004 *A*E + 2.979E-003 *B*C – 0.011 *B*D – 8.979E-003 *B*E – 0.012 *C*D - 1.646E-003 *C*E - 1.812E-003 *D*E

(4.1)

Upon conversion to actual factors, the following equation (Equation 4.2) was obtained. The conversion of the model from coded to actual factors is performed automatically by Design-Expert software as shown in Figure 5.

Lactic acid concentration g/g SPW =

-0.34330 + 3.09987E-004 *Moisture content + 7.80006E-009 *inoculum size + 0.029093 *Temperature + 0.013346 * pH + 0.013609 *Particle size + 3.46180E-011 *Moisture content *inoculum size – 3.19551E-004 *Moisture content *Temperature + 1.28125E-003 *Moisture content *pH + 3.30189E-005 *Moisture content *Particle size + 9.17584E-011 *inoculum size * Temperature – 2.2397E-009 *inoculum size *pH – 1.35670E-009 *inoculum size *Particle size – 1.78526E-003 *Temperature *pH - 1.91098E-004 *Temperature *Particle size – 1.36792E-003 *pH *Particle size.

(4.2)

The model was summarized using Diagnostic Plot to look at the normal probability plot of the residuals to check for normality of residuals (Figure 3A); residuals versus predicted values (Figure 3B) to check for constant error; Outlier T versus run order to look for outliers (Figure 3C and 3D). Evidently, the residuals fall on a straight line indicating a normal distribution of errors. Meanwhile, the plot of residuals versus the predicted value shows no observable trend and unusual structure. This observation signifies the adequacy of the model proposed by ANOVA.

Interaction between AC, AD, BC, BD, BE and CD from Figure 4 showed that there are 3 factors that influence the lactic acid metabolism, which are moisture content, inoculums size, and temperature. However, interaction between moisture content (Factor A) and temperature (Factor C) showed an obvious interaction as the moisture content was increased to 80% at high level of temperature of 40°C, the production of lactic acid were rapidly decreased.

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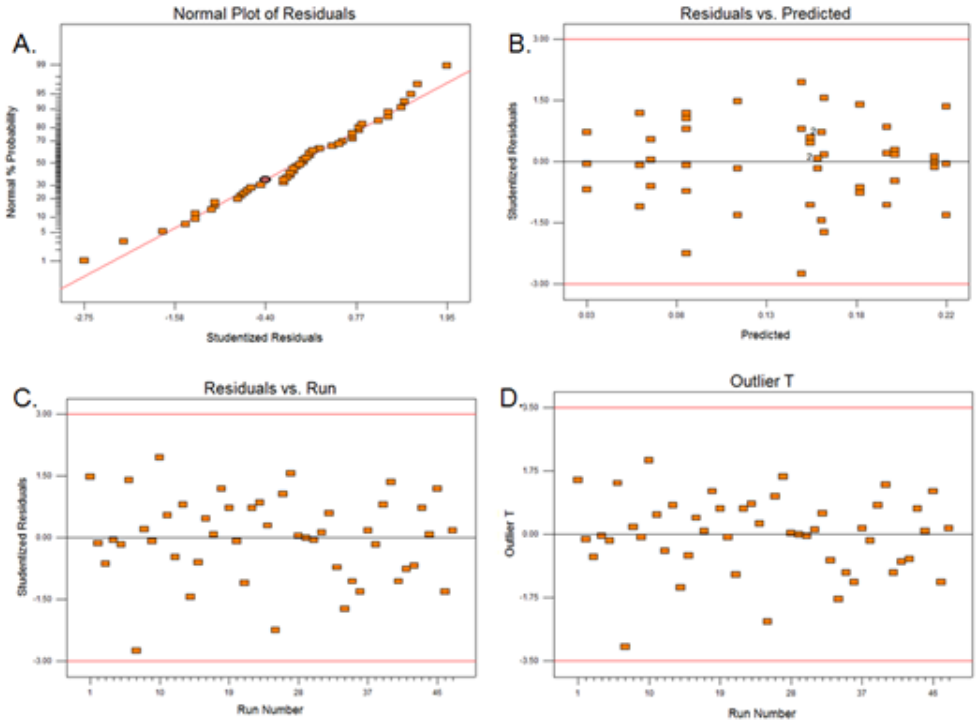

Figure 3 Summary of Diagnostic Plot. (A) Normal Probability Plot, (B) Residuals versus Predicted Values, (C) Residuals versus Run, and (D) Outlier T

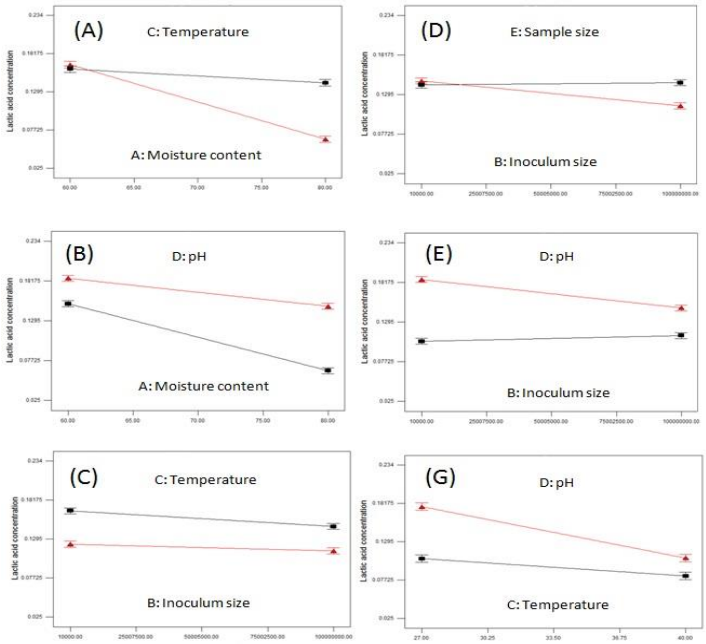

Figure 4 Summary of Interaction Graph. (A) AC, (B) AD, (C) BC, (D) BE, (E) BD, and (F) CD

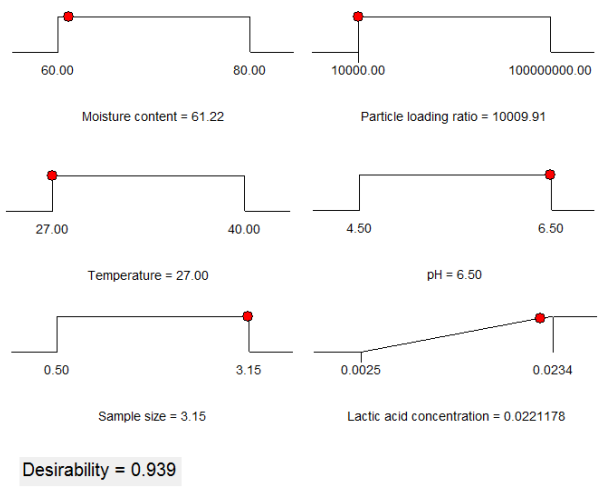

Figure 5 Optimal operating conditions suggested by Numerical Optimization Design Expert®

From the results, differences between actual and predicted values from 0.01% to 0.10% showed good precision and reliability of the experiments [19]. From previous research, Phrueksawan and his co-workers used *Rhizopus oryzae* to convert the cassava pulp into 206.20 mg/g lactic acid through SSF process [20]. Besides that, Xavier *et al*., (1994) successfully produced 5.27 g of lactic acid from 100 g of dry sugar-cane pressmed using *Lactobacillus casei subsp. casei* CFTRI 2022 [21]. However, Rojan *et al.*, (2005), have used 5 g of sugarcane baggase as a support material which impregnated with cassava starch hydrosylates in order to produce 2.9 g of lactic acid using *Lactobacillus casei* [22].

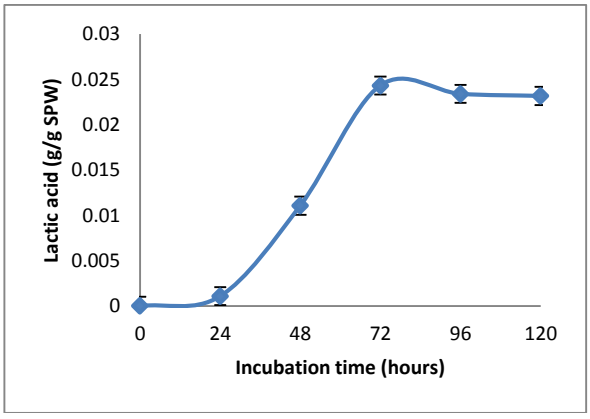

Figure 6 Production of lactic acid during 5 days (120 hours) of SSF by *Rhizopus oryzae*

The effect of incubation time during lactic acid production by *R. oryzae* in SSF was analysed. Figure 6 shows the profile of lactic acid production under the optimized condition suggested from 2 level factorial design experiments. The result illustrates that lactic acid concentration steadily increase until it reached the highest lactic acid of 0.0243 g/g SPW at day 3 (72 hours) of SSF, though long lag phase is observed (24 hours) due to the requirement of *R. oryzae* to adapt the environment prior to degrade the solid pineapple waste for growth [23]. Result obtained from the post statistical experiment has significantly confirmed the ability of *R. oryzae* to produce lactic acid at the suggested optimum condition obtained from 2 level factorial design experiments.

4.0 CONCLUSION

This study has significantly shows that *Rhizopus oryzae* was capable to utilize and convert the solid pineapple waste into lactic acid via solid-state fermentation (SSF). Confirmation run using 3.15 mm of particle size, 80% of moisture content with 1×10^4 spores/g, pH media of 6.5 and incubation temperature at 27°C gives the highest lactic acid production of 0.0236 g/g SPW with 72.89%, 69.08% and 78.98% of glucose, fructose and xylose consumption, respectively. The post-statistical experiment is almost similar with the predicted value of 0.0221 g/g SPW. Under the identified conditions, the overall productivity is 0.0009863 g/g of lactic acid concentration per hours.

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