

# LACTIC ACID PRODUCTION FROM CASSAVA MILL EFFLUENT (CME) USING *RHIZOPUS ORYZAE* IMMOBILISED IN PVA-ALGINATE SULPHATE BEADS

## Article history

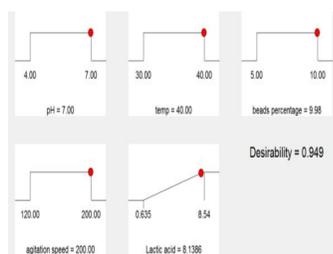
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## Graphical abstract



## Abstract

Cassava mill effluent (CME) is an effluent originated from tapioca tuber. It is produced by peeling off the skin, cutting and starch extraction. CME contains high concentration of starch, hence when it is disposed directly into the river, it will contribute to water pollution due to its high content of chemical oxygen demand (COD), biological oxygen demand (BOD), and total solids (TS). In this study, CME was used as a fermentation medium to produce lactic acid. To improve the yield, spores of *Rhizopus oryzae* were immobilised in PVA-alginate-sulphate beads and fermentation was carried out for 6 days. 2-level factorial design was used in the screening of lactic acid production for different parameters; temperature (30°C - 40°C), agitation speed (120-200 rpm), pH (4-7) and beads percentage (5-10% (w/v)). Analysis of variance (ANOVA) showed significant influence of the tested factors and their interactions on the production of lactic acid ( $p < 0.0001$ ), except for pH with ( $p = 0.0670$ ). The regression model for lactic acid production fitted the data adequately and explained the variation of more than 99% in the response. The result showed that the maximum production of lactic acid (8.54 g/L) could be achieved at the initial fermentation medium of pH 7.0, temperature of 40°C, percentage of beads of 10% (w/v) and agitation speed of 200 rpm. This study intends to exploit the potential use of CME for the production of lactic acid with the hope of contributing to Malaysia's bioeconomy.

**Keywords:** Lactic acid, cassava mill effluent, *Rhizopus oryzae*, ANOVA

## Abstrak

Cecair buangan kilang ubi kayu (CME) yang terhasil daripada proses pembuangan kulit, pemotongan dan pengekstrakan kanji daripada ubi kayu mengandungi kandungan kanji yang tinggi dan selalunya dibuang terus ke kawasan sungai berhampiran kilang. Ini boleh menyebabkan masalah pencemaran disebabkan oleh kandungan permintaan oksigen kimia, permintaan oksigen biologi dan jumlah pepejal yang tinggi dalam bahan buangan terbabit. CME digunakan sebagai medium penapaian untuk menghasilkan asid laktik. Bagi meningkatkan mutu penghasilan, spora *Rhizopus oryzae* dipegunkan di dalam manik PVA-alginat sulfat dan penapaian dilakukan selama 6 hari. Pada peringkat saringan penghasilan asid laktik, pelbagai keadaan dihasilkan menggunakan reka bentuk faktorial 2 tahap seperti suhu (30°C-40°C), kelajuan emparan (120-200 rpm), pH (4-7) and peratusan manik (5-10% (w/v)). Analisis varian (ANOVA) menunjukkan terdapat pengaruh yang besar bagi faktor-faktor yang diuji dan interaksinya terhadap penghasilan asid laktik ( $p < 0.0001$ ) kecuali faktor pH dengan ( $p = 0.0670$ ). Analisis model regresi untuk penghasilan asid laktik sesuai dengan data dengan nilai varian melebihi 99% respon. Analisis keputusan menunjukkan penghasilan maksimum asid laktik (8.54 g/L) boleh dicapai pada permulaan penapaian menggunakan pH medium 7.0, suhu 40°C, peratusan manik (w/v) 10% dan kelajuan emparan 200 rpm. Kajian ini mengeksplorasi potensi CME dalam penghasilan asid laktik yang dijangka akan menyumbang kepada bioekonomi Malaysia.

## 1.0 INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is also known as tapioca [1]. It is a type of short-term crops that are planted in Malaysia mainly to meet the demand for starch production. In 2007, the Malaysian Department of Agriculture recorded about 2,396 **hectres** of tapioca grown in Malaysia, producing about 38,711 metric tonnes valued at RM 37.5 million. In 2011, the estimated cassava planting area increased to about 2,444 hectres with potential yield of 41,045 metric tonnes. The state of Johor (mainly Johor Bahru, Kota Tinggi and Kluang districts) [2] has the largest tapioca growing area of about 875 hectre in Malaysia, and produced 19,506 metric tonnes (83% of Malaysia production) in 2005. Other states growing tapioca are Selangor (173 ha), Perak (89 ha) and Kelantan (23 ha). Most of the starch produced is used for making monosodium glutamate, and this accounts for approximately 3000 tonnes of starch per month. Other than that, tapiocas are often consumed raw and have also been made into tapioca chips [3]. Cassava wastewater or also known as cassava mill effluent is produced during the process of tapioca skin peeling, cutting and starch extraction.

CME contains a high amount of starch. It is usually disposed directly into the river and becomes a source of pollution due to its high amount of chemical oxygen demand (COD), biological oxygen demand (BOD) and total solids TS) [4]. The COD values in CME are between 33600 and 38223 mg/L whereas the BOD values are between 13200 and 14300 mg/L. Additionally, CME contains 425-1850 mg/L of free reducing sugar in the form of glucose. Other forms are hydrolysable sugars, mainly starch with the amount ranging between 22614 and 29275 mg/L. The wastewater also contains a large amount of suspended solids between 33200 and 37320 mg/L. Other substances that can be found in small traces are ashes that have values between 1450 and 1680 mg/L and nitrogen with the concentration between 97 and 182 mg/L [1].

Starch can be converted into many types of value-added products that can be used by many industries. The products produced depend on the amylolytic microorganism used. In this study, *Rhizopus oryzae* was chosen to treat CME due to its ability to convert starch into L-(+)-lactic acid in the presence of  $\text{CaCO}_3$  [5,6]. Since the global consumption of lactic acid has shown a significant increase, it is vital to find a way to produce lactic at lower cost. Hence, the utilisation of starchy raw materials such as CME can help to reduce production cost [7]. The most effective approach for L-lactic acid synthesis is through biosynthesis rather than chemical processes [6, 8]; in

fact, the only source of optically pure lactic acid isomers is from microbial fermentation [9, 10].

Lactic acid, a type of organic acid, is the precursor for poly-lactic acid (PLA). L-(+)-lactic acid is the preferred isomer of food and pharmaceutical industries since the elevated level of D-(-)-lactic acid is harmful to humans. Besides being useful in the production of poly-lactic acid, lactic acid also has numbers of other industrial applications. One of the most important applications is it can act as a preservative and acidulant in foods [11]. In addition, it also acts as a prosthetic device that controls the delivery of drugs of pharmaceutical agents and as a moisture agent in cosmetics [12, 13].

In this study, PVA-alginate-sulphate was used as the fungal immobilisation matrix. Fungal immobilisation could improve the yield of products in fermentation [14]. Various studies have employed soft gel such as Ca-alginate as a form of cell entrapment in order to immobilise *Rhizopus oryzae* for L-(+)-lactic acid production. This is because, unlike traditional fermentation, immobilisation can help solve problems such as cell wash-out at high dilution rate, higher cell concentration in the reactor, as well as cells and product separation from the system [15].

## 2.0 EXPERIMENTAL

*Rhizopus oryzae* was grown on tapioca dextrose agar. Harvested spores of *Rhizopus oryzae* then acted as an inoculum. 10 mL of inoculum of *Rhizopus oryzae* 10 mL was mixed with 90 mL of 12% (w/v) polyvinyl alcohol (PVA) and 1% (w/v) sodium alginate solution. The mixed solution of PVA-alginate and the inoculum of *A. terreus* were dropped into a mixed solution of 100 mL 5% (w/v) boric acid and 2% (w/v) calcium chloride using a syringe to form beads.

The solution was continuously stirred for 50 min. Then, the PVA-alginate-sulphate beads were kept at 4°C for 24 h. The beads were then strained, washed and stirred in 10% (w/v) boric acid solution for 30 min and further treated with 0.5 M sodium sulphate solution for another 30 min according to the method described in [16]. The beads were stored at 4°C for further use. The beads were incubated for 3 days in the growth medium at 35°C. Then, the beads were rinsed with wash medium (twice the volume of culture liquor).

Screening process of lactic acid production was investigated using 4 different variables; temperature of 30°C-40°C, agitation speed of 120-200 rpm, pH of 4-7 and beads percentage of 5% (w/v) and 10% (w/v). The fermentation study was conducted for 6 days and samples were collected daily to determine the dry

weight, starch concentration and lactic acid concentration.

Lactic acid concentration was measured using high-performance liquid chromatography (HPLC) on a Rezex ROA – Organic Acid column with the length of 300 mm and inner diameter of 7.8 mm. The mobile phase of the column was 0.005 N sulphuric acid with flow rate of 0.5 mL/min. The temperature was set at 75°C. The column was attached to the HPLC Agilent 1100 system, which was equipped with an autosampler and ultraviolet visible (UV-Vis) equipment [17].

### 3.0 RESULTS AND DISCUSSION

Screening for lactic acid production was studied using 24 runs obtained from 2-level factorial design. The purpose of the screening is to identify significant factors for lactic acid production. The selected parameters were temperature, agitation speed, pH and beads percentage.

Based on Table 1, the confidence level was greater than 95% ( $p < 0.05$ ) in lactic acid production. The F-test value of models was 255.33 and P-value was less than 0.0001. Significant interactions between model terms that affected lactic acid production were B, C, D, AB, AC and AD. The R-squared value for lactic acid production was  $0.9911 \pm 0.31$

**Table 1** Analysis of variance for lactic acid production

Source	Sum of Square	DF	Mean Square	F-Value	Prob > F	
Model	172.12	7	24.59	255.33	<0.0001	Significant
pH	0.37	1	0.37	3.86	0.0670	
Temperature	9.94	1	9.94	103.22	<0.0001	
Beads percentage (w/v)	24.49	1	24.49	306.24	<0.0001	
Agitation speed	76.71	1	76.71	796.53	<0.0001	
pH*temperature	43.08	1	43.08	447.32	<0.0001	
pH*beads percentage	8.76	1	8.76	90.97	<0.0001	
pH*agitation speed	3.77	1	3.77	39.16	<0.0001	
Pure Error	1.54	16	0.096			
Cor. Total	173.66	23				

\*A: pH

B: Temperature

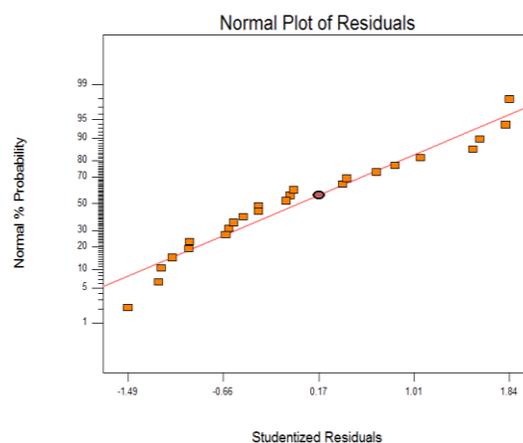
C: Beads percentage (w/v)

D: Agitation speed (rpm)

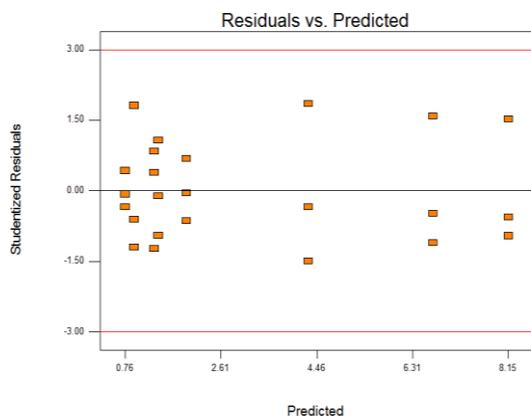
The predicted responses were correlated with experimental responses and could be observed from the R-squared, adjusted R-squared and predicted R-squared values for lactic acid production, with the values of 0.9911, 0.9872 and 0.9800 respectively.

Furthermore, the empirical model from the regression analysis of lactic acid production could be expressed by the following equation: (Lactic acid production =  $+23.88334 - 6.48621 * \text{pH} - 0.85375 * \text{temperature} - 0.44271 * \text{beads percentage} + 0.081032 * \text{agitation speed} + 0.17863 * \text{pH} * \text{temperature} + 0.16111 * \text{pH} * \text{beads percentage} - 6.60694 \text{E-}003 * \text{pH} * \text{agitation speed}$ )

In addition to ANOVA analysis, the data of lactic acid were analysed using the diagnostic tools from Design Expert. The normal plot of residual is presented in Figure 1. The residuals falls in almost a straight line, indicating there is a normal distribution of error. Meanwhile, in Figure 2, the points of residual versus predicted value of lactic acid were randomly scattered.

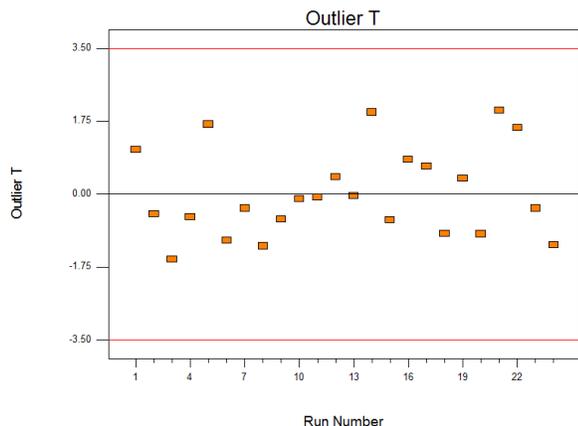


**Figure 1** Normal residuals of lactic acid production



**Figure 2** Point of residual versus predicted value of lactic acid

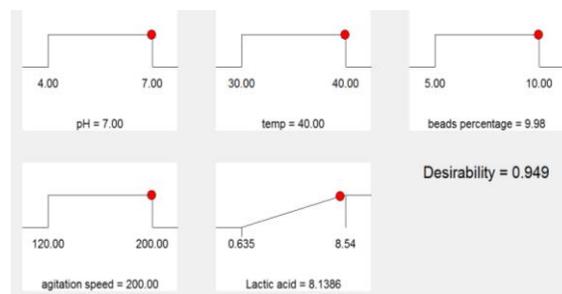
Figure 3 shows that there was no outlier and the points were distributed within the baseline and no points were out of the range.



**Figure 3** Outlier of lactic acid production

The result of the analysis showed that the maximum production of lactic acid of 8.54 g/L could be achieved at initial fermentation medium pH of 7.0, temperature of 40°C as shown in Figure 4, beads percentage of 10% (w/v) and agitation speed of 200 rpm.

The actual value of lactic acid production was compared to the optimised condition predicted by Design Expert software. The conditions are pH of 7.0, temperature of 40°C, beads of percentage 9.98% (w/v) and agitation speed of 200 rpm. The predicted lactic acid concentration was 8.1386 g/L with desirability of 0.949.



**Figure 4** Optimised condition predicted by Design Expert software

In this experiment, the role of pH was not really significant because it was only pre-set before the fermentation and was not controlled throughout the experiment. This was evident as the highest yield of lactic acid (8.54 g/L) was obtained at pH of 7 (the highest pH tested) and followed by 7.1 g/L of lactic acid production at pH of 4 (the lowest pH tested).

In the study made by Yuwa-amornpitak and Chookietwattana [18], the highest lactic acid was observed from the highest pH, in their case was pH 9. This proves that *Rhizopus* favours higher pH to produce lactic acid more efficiently. Lactic acid could be synthesised by an enzyme called lactate dehydrogenase (LDH), and this enzyme was reported to be optimum at neutral to slightly alkaline pH of 7.2 to 7.5 [19].

According to Nur Aimi *et al.*, 2011 [20], there were reports which concluded that many species of *Rhizopus* such as *R. oryzae*, *R. arrhizus* and *R. microspores* could produce lactic acid at moderated temperature (30°C) and in their study, the highest lactic acid yield of 20 g/L was obtained at 40°C. However, it has also been reported that higher temperature in the range of 40-45°C could enhance lactic acid production. In this study, the highest lactic acid yield was obtained at 40°C, similar to the finding mentioned earlier.

Agitation speed of 200 rpm has been reported by both Nur Aimi *et al.* [20] and Yuwa-amornpitak and Chookietwattana [18] as the optimum agitation speed that produced the highest lactic acid yield. Similarly in this study, the optimum agitation speed was also achieved at 200 rpm. The reason that lactic acid was produced optimally at higher agitation is probably due to high transfer rates of substrates and oxygen, which is largely due to the medium homogeneity caused by mixing [21]. Besides, an increase in agitation also increases the oxygen supply in the medium that promotes both fungal growth and lactic acid production from *Rhizopus oryzae*.

## 4.0 CONCLUSION

In this study, fungus *Rhizopus oryzae* was immobilised in PVA-alginate-sulphate beads in order to increase lactic acid production. A neutralising agent of CaCO<sub>3</sub> was added to the production medium in order to control the pH during fermentation. Effects of pH, temperature, agitation speed and beads percentage on lactic acid production were studied using the conditions derived from 2-level factorial design. The maximum production of lactic acid (8.54 g/L) was achieved with initial fermentation medium pH of 7.0, temperature of 40°C, beads percentage of 10% (w/v) and agitation speed of 200 rpm.

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