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Optimising Production of Kojic Acid from Sago Fibre by Solid-State Fermentation Using Response Surface Methodology

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ABSTRACT

Kojic acid is an organic acid that has manifold industrial applications mainly in cosmetics, food and medicine. This research aims to optimise the production of kojic acid from sago fibre using a locally isolated *Aspergillus flavus* strain under solid-state fermentation (SSF). Central Composite Design (CCD) of Response Surface Methodology (RSM) consisting of 5 factors and 5 levels were employed to determine the influence of culture conditions such as initial moisture content (50-90% (v/w)), inoculum density (5-35% (v/w)), urea concentration (0.5-3.5% (w/w)), mineral salts solution (5-35% (v/w)) and incubation time (9-21 days) on kojic acid production. The results showed that the data were best represented by a quadratic model where the significant factors for kojic acid production were identified as inoculum density and incubation time with their optimal values of 30% (v/w) and 18 days, respectively. The maximum production of kojic acid achieved in this study represented a 2-fold increase from that achieved in the non-optimised conditions. In summary, this work describes the optimisation of kojic acid production from sago fibre employing RSM. In addition, this research represents a further step towards developing a sustainable production of kojic acid employing an eco-friendly and low-cost indigenous feedstock.

Keywords: *Aspergillus flavus*, kojic acid, optimisation, Response Surface Methodology (RSM), sago fibre, sago 'hampas', solid-state fermentation

INTRODUCTION

Kojic acid is a metabolite typically produced by *Aspergillus, Penicillium* and *Acetobacter*. The most prominent role of kojic acid is found to be in cosmetic, food and health care industries as it serves as an essential depigmenting agent for production of cosmetic products (Saeedi *et al*., 2019), antioxidant agents (Burnett *et al*., 2010) and food additives. Over the decades, kojic acid has replaced the role of hydroquinone, which has been banned in many healthcare products due to its carcinogenic effects (Saeedi *et al*., 2019). This has resulted in an escalating demand for kojic acid as the alternative depigmenting agent across the globe.

Conventionally, kojic acid is produced via chemical synthesis. Nonetheless, the method is claimed as cumbersome and expensive. Hence, adoption of fermentation as a production route has become a common practice particularly at the industrial level as it is more economical and environmentally friendly. A number of works have reported the production of kojic acid via fermentations addressing various aspects (Suhaili *et al*., 2015; Liu *et al*., 2016; Shakibaie *et al*., 2018). One of the remaining bottlenecks of the industrial production of kojic acid is the expensive cost of raw materials. Although there is a number of works that reported the production of kojic acid by fermentations, nevertheless, most of the works focused on the use of synthetic media as feedstocks, which are often deemed as costly.

Utilisation of agricultural residues as alternative substrates offers an economical and eco-friendly solution as they are relatively cheaper than synthetic media apart from their wide ubiquity. It is revealed that kojic acid can be produced using starchy sources such as corn starch (Futamura *et al*., 2001), sago starch and potato starch (Rosfarizan *et al*., 1998). Among the challenges related to the utilisation of agricultural waste as feedstocks for kojic acid production in the aforementioned works, which employed

submerged fermentation (SmF) as the mode of fermentation, is the need to hydrolyse the starch before the hydrolysate can be dissolved in the liquid medium. Although the hydrolytic pre-treatment is feasible to degrade the biomass into fermentable sugars, the process is considered laborious and expensive.

Employment of solid-state fermentation (SSF) as a means for bioproductions utilising agricultural waste is seen as a better option to SmF in several aspects. Unlike SmF, the nature of SSF, which is free from flowing water, enables the direct use of agricultural waste as substrates without the need for a separate hydrolytic pre-treatment before the fermentation. Furthermore, SSF requires lower energy requirement, capital and operating costs despite being able to give comparable product yield to that achieved under SmF (Soccol *et al*., 2017). Over time, an extensive literature on SmF for kojic acid production has developed whilst little interest is given to SSF.

One of the potential agricultural residues is sago fibre or locally known as sago 'hampas', which is a by-product generated upon the processing of sago starch. Owing to its high starch content (66%) (Chew *et al*., 1993) and high abundance particularly in Sarawak, sago fibre can serve as a promising culture substrate. Currently, sago fibre is still under-utilised whereby a surplus of it is dumped into the water streams causing severe environmental pollutions. Our preliminary study has demonstrated the feasibility of bioconversion of sago fibre to kojic acid by *A. flavus* Link 44-1 via SSF where the yield was 0.27 g kojic acid produced per 1 g of sago fibre consumed (Spencer *et al*., 2011). Further optimisation of kojic acid production from sago fibre is therefore necessary where this can be accomplished by employing systematic statistical methods such as Response Surface Methodology (RSM).

The aim of this work is to optimise the production of kojic acid from sago fibre using RSM. Specifically, the study focused on the influence of culture conditions such as incubation time, initial moisture content, urea concentration, mineral salts concentration and inoculum density on kojic acid production. To the best of our knowledge, this work was amongst the first attempts to produce kojic acid from sago fibre under SSF along with our preliminary works (Spencer *et al*., 2011; Spencer *et al*., 2012). The outcomes of this work will serve as a useful insight into sustainable and economical production of kojic acid as well as further utilisation of sago fibre as a low cost feedstock for production of other value-added products.

MATERIALS AND METHODS

Microorganism

The strain used in this work namely *A. flavus* Link 44-1 was obtained from Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. Prior to use, the fungi were grown on malt extract agar (Merck, Germany) at 30 ºC for 7 days before they were harvested using 0.001% (v/v) Tween-80. The inocula used in this study were prepared in the form of spore suspension.

Sago Fibre

Sago fibre was collected from Herdsen Sago Mill in Pusa, Sarawak. The fibre was obtained by filtering the sago effluent through a 1 mm stainless steel filter at the sago mill discharge point before it was then transported to the laboratory and stored at -20 °C. Prior to use, the fibre was oven-dried and ground to particle size of approximately 1 mm.

Solid-state Fermentation

In every fermentation, 5 gm of sago fibre was added to a petri dish and it was then supplemented with urea and mineral salts solution consisting of 0.2% (w/v) potassium dihydrogen phosphate, 0.05% (w/v) magnesium sulphate heptahydrate and 1% (w/v) yeast extract. All fermentations were conducted at 30 °C. The parameters of interest for fermentations were initial moisture content [50-90% (v/w)], inoculum size [5-35% (v/w)], mineral salts concentration [5-35% (v/w)], urea concentration [0.5-3.5% (w/w)] and incubation time (9-21 days). Besides being typical parameters studied in any SSF-based processes, the aforementioned parameters and their ranges were determined upon the screening stage in our preliminary study (Spencer, 2015). The cultures were sampled every two days. The sampled culture was mixed with 40 mL of distilled water before the suspension was centrifuged at 6000 rpm and 4 °C for 20 minutes (Conti *et al*., 2001) and filtered

through a sterile Minisart 0.45 µm filter.

Kojic Acid Analysis

The analysis of kojic acid in this work was based on a method by Ariff *et al*. (1996) with some modifications. Kojic acid was quantified using High Performance Liquid Chromatography (HPLC) (Shimadzu, Kyoto, Japan) with ultraviolet detector at 260 nm. The HPLC was equipped with Shimadzu Refractive Index Detector (RID-10A). The mobile phase used was 5 mM H_2SO_4 with a flow rate of 0.8 mL/min at 60 ^oC while the stationary phase was Bio-Rad Fermentation Monitoring Column (150 mm x 7.8 mm). Commercial kojic acid (Sigma, USA) was used as a standard with the concentrations used ranging from 0.02 to 0.1 g/L. The retention time of kojic acid was found to be 4.11 min.

Experimental Design and Statistical Analysis

In the preliminary studies, a 5-level/5-factor Central Composite Design (CCD) was used. The independent variables were initial moisture content (A), inoculum size (B), mineral salts concentration (C), urea concentration (D) and incubation period (E). The dependent variable (response) was kojic acid concentration. The independent variables were studied at five different levels: negative alpha (-α), negative one (-1), zero (0), one (1) and positive (+α) as shown in Table 1.

The interaction among the factors and the response was investigated by using the following model:

$$Y = \beta_o + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i \neq j=1}^{n} \beta_{ij} x_i x_j + \epsilon \quad (1)$$

where,

Y = Predicted response
β_o = Value of the fixed response at the center point of the design
$\beta_i, \beta_{ii}, \beta_{ij}$ = Linear, quadratic, and interaction effect (cross product coefficients) regression terms, respectively
x_i, x_j = Independent variables (factors)
ϵ_I = Error

A statistical software package by Design Expert Version 8.0.1 (Stat-Ease Inc., Minneapolis, USA) was used to analyse the design matrix data and to generate the response surface graphs. A total of 50 experiments comprising of 32 factorial experiments (levels -1, 0, and +1), 10 axial experiments (levels ±α), and 8 replicates in the centre point were conducted. In addition, 5 control experiments were added to monitor the influence of natural attenuation. An analysis of variance (ANOVA) was performed to determine the fitness of the model and its statistical significance.

RESULTS AND DISCUSSION

Model Fitting and Statistical Analysis

Five important culture parameters namely, initial moisture content (A), inoculum density (B), incubation period (C), concentration of urea (D) and concentrations of mineral salts (E) were identified as the independent variables and kojic acid production was the dependent variable (response). Initial analysis showed that initial moisture content, urea concentration and mineral salts concentration gave insignificant effects ($p>0.05$) on kojic acid production whilst other factors showed positive effects. Hence, the three aforementioned factors were excluded from the subsequent analysis. Regression analysis of the data showed that the data were best described by a quadratic model as follows:

$$Y = 1.71 + 0.26B + 0.19B^2 + 0.13C^2 \quad (2)$$

where,
Y = Kojic acid concentration
B = Inoculum density
C = Incubation period

Table 2 outlines the ANOVA analysis of the RSM model after the exclusion of the insignificant factors. The analysis showed that the suggested model was significant ($p=0.02<0.05$) with 95% confidence interval. The model terms B, B^2 and C^2 were significant in terms of kojic acid production. Meanwhile, the p-value of lack-of-fit (0.2233) indicates the insignificance of lack-of-fit with respect to the pure error. This consequently implies a good fitting of the data to the suggested model. The significance of the suggested model was determined by the value of determination coefficient (R^2), which showed that 59.86% of the variability is attributed to the predicted model.

Model verification was carried out by comparing the experimental and predicted response at optimum conditions as outlined in Table 3. The error represents the deviation between the predicted and the actual kojic acid

Table 1. Variables with their levels selected for kojic acid fermentation.

Variable	-α	-1	0	+1	+α
Initial moisture content (% (v/)) (A)	50	60	70	80	90
Inoculum size (%) (B)	5	10	20	30	35
Mineral salts concentration (% (v/w)) (C)	5	10	20	30	35
Urea concentration (% (w/w)) (D)	0.5	1.0	2.0	3.0	3.5
Incubation period (Days) (E)	9	12	15	18	21

Table 2. Analysis of variance (ANOVA) for the regression model of kojic acid production from sago fibre by *A. flavus* Link 44-1.

Source	**Sum of Squares**	**DF***	**Mean Square**	**F-Value**	**P-value**
Model	6.78	20	0.34	2.28	0.0210
B	2.96	1	2.96	19.91	0.0001
B^2	1.95	1	1.95	13.13	0.0011
C^2	0.90	1	0.90	6.03	0.0203
Residual	4.31	29	0.15		
Lack of Fit	3.66	22	0.17	1.77	0.2233[a]
Pure Error	0.66	7	0.094		
Cor Total	11.10	49			
R^2	0.5986				

Table 3. Verification of experiments carried out at optimum conditions.

Run	Initial moisture content (%) (v/w)	Inoculum density (%)	Incubation period (Days)	Urea (%) (w/w)	Mineral salts (%) (v/w)	Predicted kojic acid (g/L)	Actual kojic acid (g/L)	Desirability	Error
A	80.00	30	18	1.70	30	2.70	2.71	0.62	0.52
B	79.61	30	18	2.34	30	2.68	2.68	0.62	-0.30
C	80.00	30	18	2.59	30	2.68	2.75	0.62	2.30
D	80.00	30	18	2.72	30	2.68	2.65	0.61	-1.13
E	77.54	30	18	1.48	30	2.67	2.70	0.61	1.33

concentration. In general, all the five treatments yielded errors of below 5% suggesting the reliability of the predicted model in elucidating the relationship between kojic acid production and its two significant factors: inoculum density and incubation period.

Analysis of Response Surface Methodology (RSM)

Response surface plots were used to investigate the interactive effect of the two significant factors (inoculum density and incubation period) on kojic acid production. Figure 1 plots the interactions between the inoculum density and incubation period and their effects on kojic acid production. In this treatment, the initial moisture content, urea and mineral salts of the cultures were kept at 80% (v/w), 2.59% (w/w) and 30% (v/w), respectively. It is shown that increasing both the inoculum density and incubation period resulted in an increase of kojic acid production. The maximum kojic acid production (2.75 g/L) was achieved on Day 18 when the inoculum density was 30% (v/w). Increasing the incubation time to 21 days and inoculum density to 35% (v/w) did not give any significant difference in the kojic acid titre. The maximum kojic acid concentration as achieved in this study represents a 2-fold increment in comparison to the maximum kojic acid achieved in non-optimised fermentations as reported in our preliminary study by Spencer *et al*. (2011).

In many SSF-based processes, inoculum density plays a pivotal role in determining the maximum productivity of the cultivation. Although one of the challenges of SSF lies in the uniformity of the inoculum grown on the

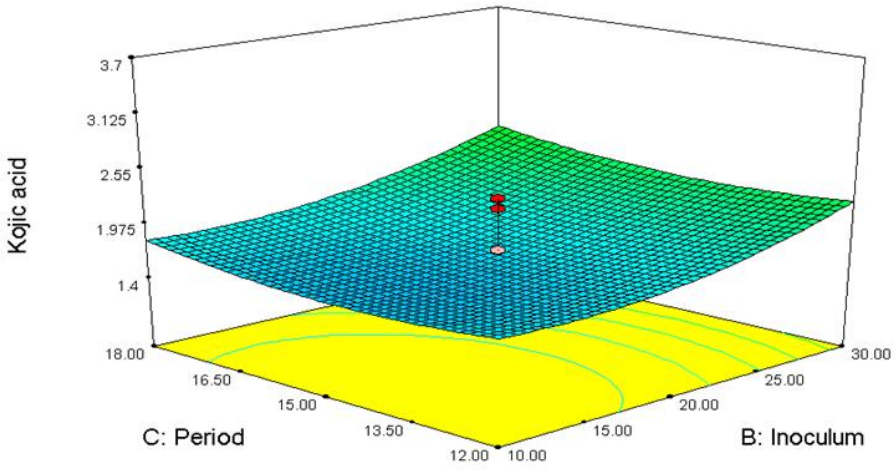

Figure 1. Response surface three dimensional (3D) plot indicating the interaction between inoculum density and incubation period and their effects on production of kojic acid from sago fibre by *A. flavus* Link 44-1.

substrates, this may be partly alleviated by applying an optimal density of inoculum that can ensure its thorough distribution throughout the substrate thus preventing undesirable competition for nutrients. As in this work, optimal inoculum density can facilitate optimal secretion of enzymes responsible for degradation of lignocellulose and starch to fermentable sugars, which are in turn converted to kojic acid.

It is worth noting that *A. flavus* Link 44-1 in this study was able to feed on the non-treated sago fibre to eventually produce kojic acid. This is probably due to the ability of the strain to produce amylolytic enzymes such as glucoamylase and α-amylase that are essential for degrading starch into simpler sugars (Rosfarizan *et al*., 1998). This strongly implies the promising use of the strain for SSF that employ starchy sources as substrates. Contrary to the case if sago fibre is to be used in SmF-based processes, hydrolytic pre-treatment is vital in order to degrade the starch into fermentable sugars. This has been demonstrated by Awang-Adeni and co-workers (2013) in their work on the utilisation of sago fibre for bioethanol production via SmF. Elimination of the hydrolytic pre-treatment of sago fibre as shown in this work, has made SSF as a more attractive means for kojic acid production than SmF-based processes.

The optimal incubation time for kojic acid production as found in this study (18 days) was relatively longer than the range that was reported for batch SmF-based processes in some of prior research, which were between 8 and 15 days (Ariff *et al*., 1996; Rosfarizan *et al*., 2002; Shakibaie *et al*., 2018). The length of the optimal incubation time may be attributed to the type of media used as well as the types and modes of fermentation employed. Most of the preceding works were using synthetic media whilst the fermentation mode employed was batch SmF. The longer incubation time needed by SSF-based processes in producing kojic acid from sago fibre can be perhaps compensated by the elimination of the hydrolytic pre-treatment of sago fibre as needed in SmF-based processes. The natural degradation of lignocellulose and starch in sago fibre facilitated by amylolytic enzymes secreted by *A. flavus* during the fermentation may require an additional time for the conversion of fermentable sugars to kojic acid. This is in contrast to the fermentations that employ synthetic media as reported by the aforementioned studies where by direct conversion of the readily available sugars in the media to kojic acid may shorten the time taken to reach the maximum titre of kojic acid. According to Awg-Adeni *et al*. (2013), the structure of sago fibre comprises of starch granules that are enclosed by lignocellulosic compounds. It is suggested that the sugars used for kojic acid production in this study were originated from both lignocellulosic and starchy components of sago fibre. In-depth studies pertaining to the enzymes released by *A. flavus* Link 44-1 and their activities in degrading lignocellulose and starch in sago fibre

are therefore necessary.

CONCLUSION

In summary, this research work has demonstrated the feasibility of optimising the production of kojic acid by *A. flavus* Link 44-1 using sago fibre as a feedstock via SSF. Our results showed that a quadratic model was sufficient to describe a relationship between kojic acid production and its significant factors: inoculum density and incubation time. The best conditions of both factors were identified at 30% (v/w) and 18 days, respectively, which led to a 2-fold improvement of kojic acid production over the titre achieved under the non-optimised conditions. Future work should consider comprehensive studies on various aspects such as characterisation of enzymes responsible for lignocellulose and starch degradation in sago fibre prior to kojic acid biosynthesis. Apart from that, it is also worth to investigate the strategies for scaling up the production of kojic acid from sago fibre by SSF, where this is imperative for its commercialisation in future. From a broader context, this work provides useful insights into the development of cost effective and sustainable production of kojic acid as well as exploitation of sago fibre as a cheap SSF feedstock for production of value-added products.

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