Research Paper

Optimization of Antifungal Activity and Determination of Antifungal Mechanism in Lacto-Fermented Palm Kernel Cake Against *Colletotrichum gloeosporioides*

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Abstract

Anthracnose disease caused by *Colletotrichum gloeosporioides* reduces the postharvest quality of fruits and vegetables which creates large economic losses. Natural preservation methods of fruits have been popular due to the preference of modern consumers to consume chemical-free fruits. Therefore, there is a requirement for industrial-level natural preservation techniques for fruits and vegetables. The current study aimed to optimize the antifungal activity of previously produced fungal growth inhibitor (PKCL1) by *Lactobacillus plantarum* cultivated in palm kernel cake (PKC) against *Colletotrichum gloeosporioides* and to determine the antifungal mechanism. Box Behnken design under response surface methodology was applied to optimize the fermentation conditions. The antifungal mechanism of optimized PKCL1 was determined by the leakage of intercellular components, ergosterol synthesis, and the morphological changes of treated fungi. The substrate ratio, fermentation time, and temperature influenced the production of antifungal compounds while altering the antifungal activity. The combinations of optimum fermentation conditions comprised of substrate ratio of 24.75%, fermentation time of 96 hours, and fermentation temperature of 37 °C to obtain 88.1% *C. gloeosporioides* inhibition. Significant differences were observed in the protein concentration, DNA, sugars, and ergosterol synthesis between the treated and non-treated fungi. Thus, PKCL1 was affected in the cell membrane structure and biosynthesis pathways of *C. gloeosporioides*. Besides, PKCL1 changed the cell morphology and destroyed the cell wall structure. Box Behnken design is an appropriate model to optimize the fermentation conditions to maximize the antifungal activity of PKCL1. It was concluded that optimized PKCL1 has a strong capability to destroy *C. gloeosporioides.*

Keywords: Anthracnose, Antifungal activity, Antifungal mechanism, Optimization, RSM

Introduction

Anthracnose caused by *Colletotrichum gloeosporioides* spp. is known as one of the major fungal diseases of fresh fruits and vegetables. Synthetic fungicides are commonly used as component of anthracnose disease control. (Mahajan *et al*., 2014).Consumers prefer safer agricultural produce and are more likely to reject the fresh produce treated with chemical fungicides, and green-labeled fresh produce motivates consumer demand and social acceptance. (Prusky, 2011). Bio-preservation is an alternative technology for controlling the growth of harmful microorganisms that is safe for consumers and the environment (Lamont *et al*., 2017; Muhialdin, *et al*., 2020a). Lactic Acid Bacteria (LAB) has long been used as a bio preservative agent in food, and the use of LAB metabolites as bio preservatives showed significant results against a wide range of fungi. Besides, these compounds improve the postharvest life, quality, and sensory properties of various fruits (Barrios-Roblero *et al*., 2019; Marín *et al*., 2019).

Several factors influence the production of antifungal compounds via the LAB fermentation process (Lacto-fermentation) including substrate, LAB strains, temperature, pH, and fermentation time (Janakiram *et al*., 2004; Sridevi *et al*., 2015). Previous studies have reported that *Lactobacillus plantarum* has high potential for producing strong antifungal compounds. Control of these fermentation conditions within an effective range is significant in maximizing the production of antifungal compounds and reducing production costs at an industrial level (Ricci *et al*., 2019; Singh *et al*., 2017).

It has been confirmed that Response Surface Methodology (RSM) is one of the robust models, that can be utilized to decide the impact of considered factors and how the factors interact, which allows the process of optimization to be performed efficiently (Chen *et al*., 2015). The significant advantage of the RSM is that the amount of data required to estimate, analyze, and optimize the applicable parameters reduces the number of experiments needed considerably. Thus, the RSM is more rapid and inexpensive to gather refresh outcomes than traditional variables or full factor experimentation methods. Therefore, this study aimed to optimize the fermentation conditions to obtain the maximum antifungal activity for the fungal growth inhibitor produced by palm kernel cake (PKC) fermented with *L. plantarum* ATCC8014 using the RSM. In addition, the related antifungal mechanism of optimized fungal growth inhibitors against *C. gloeosporioides* was determined.

Materials and Methods

High-quality expeller-pressed palm kernel cake (PKC) was taken from the Malaysian Palm Oil Board (MPOB), Bandar Baru Bangi, Selangor, Malaysia. The pure LAB strain, *L. plantarum* ATCC8014, was obtained from the Laboratory of Food Bioprocessing, Faculty of Food Science and Technology, Universiti Putra Malaysia. *C. gloeosporioides* DSM62136 was bought from the German collection of microorganisms and cell

cultures (DSMZ), Braunschweig, Germany. LAB culture was grown in De Man, Rogosa, and Sharpe (MRS) broth (pH 6.5) (Sigma-Aldrich), and the homogenous suspension was adjusted to obtain 1 × 107 CFU mL^{-1}. *C. gloeosporioides* DSM62136 was grown on PDA (OXOID, Columbia, USA) at 30 °C and the conidia concentration was adjusted to 1 × 10^6 conidia mL^{-1} (Abril *et al*., 2008) using a hemocytometer (A3835, HEMC, Noida, India).

The fermentation substrate (PKC) was prepared using the previously described method by Erukainure *et al*. (2010) with minor modifications. PKC was cleaned and homogenized at the ratio of 30%. Samples were sterilized by autoclaving at 121 °C for 15 minutes. Thereafter, 2% (v/v) of LAB inoculum was added to the fermentation mixtures and they were incubated under an anaerobic condition with mild agitation (180 rpm). Fermented samples were sterilized again at 121 °C for 15 min. to deactivate the microorganisms and enzymes. The cell-free supernatant (CFS) samples were produced using a centrifuge at 5000 × g for 10 min (Sigma 3-18KS, Germany).

Preliminary optimization studies

In a previous study (Ranjith *et al*., 2021), the antifungal activity of the fungal growth inhibitor produced by *L. plantarum* cultivated in PKC has been confirmed. To maximize antifungal activity, the procedure was optimized for one specific variable and its levels were included in the optimization procedure.

Determining of response parameters

Antifungal activity of CFS was determined by a liquid system using a 96 microwell assay as described by Lavermicocca *et al*. (2003). The volume of 100 µL malt extracts broth (MEB) containing 5 µL of 1 × 10^6 conidia mL^{-1} suspension was added to the wells. The wells were then filled with 150 µL of prepared CFS samples. (25 mg mL^{-1}). The fungal development was measured at OD600 using an Elisa reader (Bio-RAD 170-6930, Hercules, California, USA) at 30 °C for 0 h and 72 h. Antifungal activity of CFS samples was defined as the percentage of fungal growth inhibition, and that was calculated using the following formula:

$$\text{Inhibition}(\%) = \frac{(72\text{ h control-0 h control}) - (72\text{ h treatment -0 h treatment})}{(72\text{ h control - 0 h control})} \times 100$$

The total peptide concentration was determined by the Ophthaldialdehyde (OPA) assay, previously defined by Goodno *et al*. (1981). Diluted CFS samples (27 µL) and 205 µL of prepared OPA solution were added into a microwell. Deionized water and an OPA solution were used as the control. Peptide content of the samples was measured at 340 nm using an Elisa reader. The peptide concentration was estimated against the standard curve of L-serine (10 mM to 0.3125 mM).

The pH values of the fungal growth inhibitor samples were determined using a bench top digital pH meter (Fisherbrand accumet™ AE150, Leicestershire, UK).

A modified 96 microwell Folin-Cioalteu assay, as described by Zhang *et al*. (2006), was used to measure the total phenolic content with slight modifications. The microwells were filled with 20 µL of ten-fold diluted CFS samples and 100 µL of Folin-Cioalteu regent. After 30 minutes of incubation in the dark condition 80 µL of a 7.5% Na_2CO_3 solution was added.. Absorbance was recorded at 750 nm. The standard curve was prepared using gallic acid series (0- 600 µg mL^{-1}). Each sample was tested in triplicate.

Determination of the antifungal mechanism

With minor modifications conidia germination inhibition was determined as described by Chen *et al*. (2018). First, 20 µL of *C. gloeosporioides* fungal conidia suspension (1 × 10^6 conidia mL^{-1}) was incubated with two different concentrations of PKCL1 in PDA (10 mL), namely the minimal inhibition concentration (MIC) (20 mg mL^{-1}) and 50% of MIC (10 mg mL^{-1}) .A sample without PKCL1 was used as the control. The cultured plates were incubated at 28°C for 15 h and homogenized. 10 µL spore suspension was placed in a depression slides and the number of germinated conidia was assessed. At least 200 conidia were considered in each visual field to count. All the tests were performed in triplicate.

C. gloeosporioides mycelia for further assays were prepared as described previously by Chen *et al*. (2018). Fungal conidia were cultured on potato dextrose broth (PDB), and mycelium was harvested carefully. The mycelia was lyophilized at -46°C (FDU-1200, EYELA, Tokyo, Japan). Two mg lyophilized mycelium were re-suspended in 0.1 mol L^{-1} PBS (pH 7.0) and added to the optimized PKCL1 concentration at MIC (20 mg mL^{-1}). The identical mycelia sample with the addition of the commercial fungicide, Benomyl (0.085 mg mL^{-1}), was positive control (Abril *et al*., 2008). Fungal suspension with only PBS was used as the negative control.

Leakage of total soluble protein, total sugars, and DNA of treated and non-treated fungi samples were measured by the Bradford assay (Nouroozi *et al*., 2015), the anthrone sulfuric acid method (Moshayedi *et al*., 2013), and the spectrophotometric method (Chen *et al*., 2018), respectively. The Bradford assay was performed in microwells with 10 µL of sample and 190 µL of ready-to-use Bradford reagent (B6916, Sigma- Aldrich, Saint Louis, USA) at 595 nm. The absorbance was measured at 625 nm using a microplate reader to measure total sugars under the anthrone sulfuric assay. DNA related absorbance was measured at 260 nm using a UV visible spectrophotometer (BIOMATE 3, Thermo Fisher Scientific, United States).

The ergosterol concentration was measured as the method previously described by Wang *et al*. (2019) with slight modifications. Three mg lyophilized mycelium were re-suspended in 0.1 mol L^{-1} PBS (pH 7.0) and added PKCL1 concentration at MIC (20 mg mL^{-1}). Samples were incubated at 28 °C

with mild (120 rpm) agitation. After that, the fungal precipitate was collected, and 10 mL of 20% sodium hydroxide solution and 5 mL of 96% ethanol were added. In a hot water bath, the solution mixture was saponified at 90 °C for 1.5 h two times. Petroleum ether (10 mL) was added after cooling to room temperature, and the solution was kept on a horizontal shaker for 20 min. The mixture was let stand for 30 min to allow for extraction, and the step of extraction was done twice.

Morphological changes in treated and untreated fungal mycelia of *C. gloeosporioides* were observed using scanning electron microscopy (SEM) (JSM-6400; JEOL) as the method described by Da *et al*. (2019). The conidia suspensions (100 µL) were inoculated on PDA media containing PKCL1 with a concentration of 25% MIC (preliminary studies). The cultured plates were incubated at 28°C for 4 days to collect the fungal mycelia. Mycelia samples were mounted on silver paste (Nisshin EM, Tokyo, Japan) and sputter-coated with gold using an ion coater. Samples were observed by the SEM (JSM-6400; JEOL, Tokyo, Japan) at a voltage of 15 kV.

Experimental design and data analysis

Box-Behnken design for Response Surface Methodology (RSM) was conducted for the optimizing procedure to study the combined effect of the substrate ratio, fermentation time, and temperature on antifungal activity, pH, total peptide concentration, and phenolic concentration over three coded levels (low (-1), medium (0), and high (+1)) during favor 15 runs (Table 1) (Bosque-Sendra *et al*., 2001).

Table 1. Coded and actual levels of the variables for three-factor Box-Behnken design

Independent variables	Symbols	Coded and actual levels		
		-1	0	+1
Substrate ratio (%)	X_1	10	20	30
Fermentation time (h)	X_2	48	72	96
Temperature (°C)	X_3	31	34	37

Optimization of variables: The optimizing design was developed using Minitab 17 (Minitab LLC, PA, and USA) statistical software and resulted in 15 experimental runs (Table 2). The levels of independent variables were designated based on preliminary experiments. The second-degree polynomial of the design was expressed using the following formula.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_{12} + b_{22}X_{22} + b_{23}X_{32}$$

Where: Y is predicted response, X_1, X_2, and X_3 are independent variables, b_0 is offset term, b_1, b_2, and b_3 are the linear effects, and b_{11}, b_{22}, and b_{13} are the interaction terms.

Statistical significance of the regression equations was tested using an F-test. The ANOVA was performed to determine whether the quadratic models were significant. *P*-values were used to check the significance of coefficients, which reflected the pattern of the interactions between independent variables. The following second-degree polynomial was determined using the multiple regression analysis on the experimental data to represent the correlation between the response

parameters and fermentation conditions by the acquired second-order polynomial equations. After the significance of the experimental values of parameters has been assessed, the model can be developed by reducing the terms that are not significant levels ($P< 0.05$).

Optimization and validation conditions: Multiple graphical optimizations were accomplished by overlaying all 3D plots. Several numerical optimizations were also carried out to achieve the best overall fermentation conditions. The overlaid plot was drawn to visualize the significant ($P<0.05$) interaction effect of fermentation conditions on the response parameters. The various numerical optimizations were carried out to determine the best fermentation conditions and attain the desired results.

Determination of cell wall composition and intercellular compounds: A randomized complete block design (RCBD) was used to experiment with changes in intercellular compounds and the cell wall composition of fungal cells. The data were subjected to analysis of variance (ANOVA) while using the Statistical Analysis System (SAS), version 9.4 (SAS Institute Inc., Cary, NC, USA). The means were compared using the Tukey multiple comparison test at the significance level of $P<0.05$.

Results and Discussion

Optimization of variables

The RSM has optimized three independent variables: substrate ratio, fermentation time, and temperature. The final models for explaining the relationship between the response parameters and the independent variables are shown in Equations 1-4. The regression equations' observed and predicted values are presented in Table 2.

$$Y_1=6.375 - 0.03150X_1 - 0.00464X_2 - 0.0537X_3 \quad \text{-Eq. 1}$$

$$Y_2=2396+8.78X_1+0.674X_2-106.2X_3-0.1916X_12+1.582X_32 \quad \text{-Eq. 2}$$

$$Y_3=1111+0.387X_1-1.340X_2-61.0X_3+0.01045X_22+0.916X_32 \quad \text{-Eq. 3}$$

$$Y_4=608 +0.380X_1+0.2009X_2 -33.1X_3 +0.495\ X_32 \quad \text{-Eq. 4}$$

The significance of the fit of the second-order polynomial for the pH value, total phenolic content, peptide content, and percentage of *C. gloeosporioides* inhibition was evaluated by implementing the analysis of variance (ANOVA) (Table 3). The small *P*-values of main effects are, the bigger the significance of the corresponding coefficient, which suggests that the model is suitable for the optimization (Tables 3). As a result, a slight variation can alter the pH value, total phenolic content, peptide content, and percentage of *C. gloeosporioides* inhibition to a considerable extent.

Table 2. Box- Behnken design for optimization of fermentation conditions of PKC fermented with *L. plantarum* and the observed values of response variables

	Variables									
	Coded			Process			Response variables			
Run	X_1	X_2	X_3	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4
1	-1	-1	0	10	48	34	3.98	715.46	58.36	65.58
2	+1	-1	0	30	48	34	3.51	759.73	73.97	77.84
3	-1	+1	0	10	96	34	4.07	748.78	66.56	75.71
4	+1	+1	0	30	96	34	3.23	760.79	75.36	79.87
5	-1	0	-1	10	72	31	4.14	747.47	65.67	75.29
6	+1	0	-1	30	72	31	3.32	756.64	67.23	78.70
7	-1	0	+1	10	72	37	3.62	742.55	72.62	71.35
8	+1	0	+1	30	72	37	3.23	766.45	77.61	81.92
9	0	-1	-1	20	48	31	3.93	743.40	64.37	70.06
10	0	+1	-1	20	96	31	3.47	801.52	80.40	89.24
11	0	-1	+1	20	48	37	3.48	768.29	76.12	82.65
12	0	+1	+1	20	96	37	3.24	805.12	82.20	89.88
13	0	0	0	20	72	34	3.82	759.46	63.26	77.71
14	0	0	0	20	72	34	3.37	757.02	61.65	76.66
15	0	0	0	20	72	34	3.35	754.54	59.11	74.65

Three process variables substrate ratio (%) (X_1), fermentation time (h) (X_2) and fermentation temperature (°C) (X_3) with response variables; pH (Y_1), total phenolic content (μg GAE 100 mL^{-1}) (Y_2), peptide content (μg mL^{-1}) (Y_3), and percentage of C. gloeosporioides inhibition (Y_4), to optimize the fermentation conditions of PKC with L. plantarum.

Table 3. Regression coefficients (RC) and significant *P*-values (P) of estimated second-order reduced response models

		Y_1		Y_2		Y_3		Y_4	
		RC	*P*	RC	*P*	RC	*P*	RC	*P*
Linear	C	3.584		761.78		62.030		75.440	
	x_1	-0.315	<0.001	11.170	0.025	3.870	0.013	3.800	0.037
	x_2	-0.111	0.014	16.170	0.004	3.960	0.012	4.820	0.012
	x_3	-0.161	0.026	14.170	0.034	3.860	0.013	1.560	0.046
Quadratic	x_1^2	-	-	-19.560	0.012	-	-	-	-
	x_2^2	-	-	-	-	6.020	0.010	-	-
	x_3^2	-	-	14.240	0.045	8.240	0.002	8.910	0.083
Interaction	x_1x_2	-	-	-	-	-	-	-	-
	x_1x_3	-	-	-	-	-	-	-	-
	x_2x_3	-	-	-	-	-	-	-	-
Lack of Fit	(LF)		0.905		0.330		0.244		0.093
	R_2	0.76		0.81		0.86		0.66	

Across the row; coefficient (C), second-order linear effect of substrate ratio (X_1), fermentation time (X_2), fermentation temperature (X_3) quadratic effect of substrate ratio (X_1^2), fermentation time (X_2^2), fermentation temperature (X_3^2), the interaction effect of substrate ratio and fermentation time (X_1X_2), substrate ratio and fermentation temperature (X_1X_3), fermentation time and fermentation temperature (X_2X_3) and coefficient of determination (R_2) values. Across the column; pH (Y_1), total phenolic content (Y_2), peptide content (Y_3), and percentage of C. gloeosporioides inhibition (Y_4).

The result of optimization (Table 3) shows insignificant 'lack of fit' values for response parameters. These imply that it is insignificant as compared to the pure error and that the model equation was adequate for predicting the pH, total phenolic content, peptide content, and percentage of *C. gloeosporioides* inhibition. The fitness of the models was further confirmed by satisfactory values of the coefficient determination (R^2). These R^2 values indicate that 76, 81, 68, 86, 66, and 87% of the responses' variability was predicted by the models. As well, the settlement between pH, total phenolic content, peptide content, and percentage of *C. gloeosporioides* inhibition values predicted by the final models and the experimental (observed) values is substantial, as shown by a higher R^2 value.

The surface plots (Figures 1A, 1B and 1C) provided the interactions among the independent variables. They imagined the combined effects of independent variables on the response parameters. The interactions between the critical variables have been described below by the shapes of the surface plots. Figure 1A shows that the pH value decreased with the substrate ratio, temperature, and fermentation time increment. The interactions between the variables, such as the higher substrate ratio, temperature, and fermentation time supported to get low pH values.

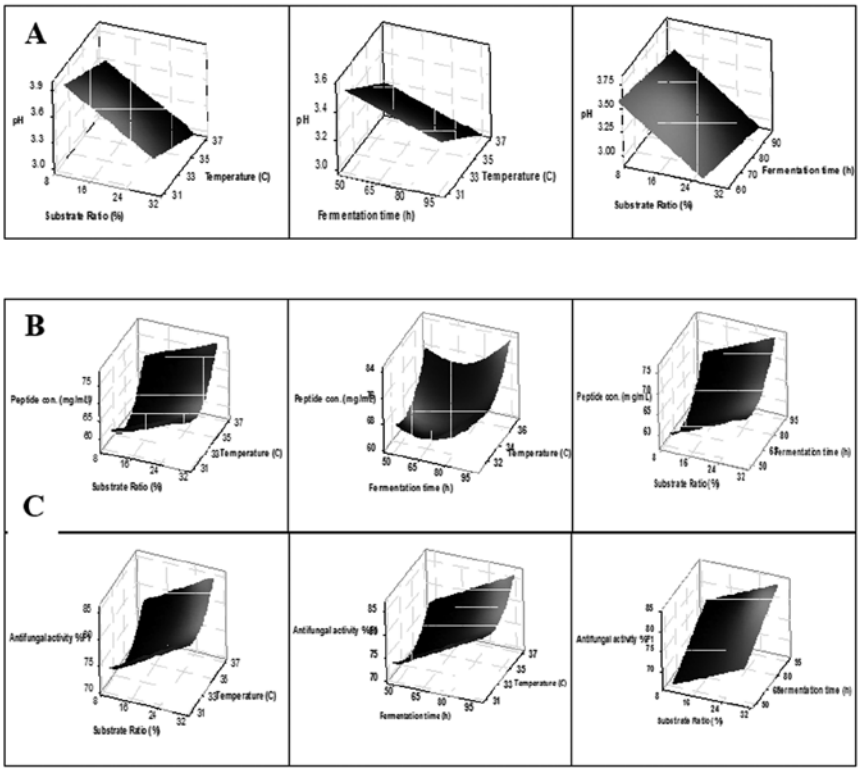

Figure 1. The interaction effects of substrate ratio (%), fermentation time (h), and temperature (°C) on pH value (A), total peptide concentration (B), and antifungal activity against *C. gloeosporioides* (C).

Also, Figure 1B shows that the peptide concentration increased with substrate ratio, temperature, and fermentation time. A higher substrate ratio increases peptides' production due to increasing the availability of protein and amino acids (Pessione and Cirrincione, 2016). In addition, enzymatic activities can be affected by bioactive peptide production; the factors affecting production are strain-specific and depend on the proteolytic system (Savijoki *et al*., 2006). The higher peptide concentrations observed at a higher temperature (37°C) coincide with the optimal temperature level of many enzymes.

As shown in Figure 1C, the antifungal activity of PKC fermented with *L. plantarum* was increased with the substrate ratio, temperature, and fermentation time during the ranges investigated. Although the increase of antifungal activity with substrate ratio was limited, and it started to reduce after the substrate ratio of 24.75%. The maximum antifungal activity against *C. gloeosporioides* was observed with the substrate ratio of 24.75% (w/v), fermentation time of 96 h, and fermentation temperature of 37 °C. Generally, the increase of antifungal activity with an increment of substrate ratio, fermentation time, and fermentation temperature could result from an increase in the production of organic acids and bioactive peptides. (Rizzello *et al*., 2011).

Optimization and validation conditions: Optimally, the fermentation conditions were 24.75% of the substrate ratio, 96 h of fermentation time, and 37 °C of fermentation temperature (Figure 2). Under the optimum conditions, the corresponding predicted response values for pH, total phenolic concentration, peptide concentration, and percentage inhibition of *C. gloeosporioides* were 3.16, 797.34, 85.95, and 88.08%, respectively.

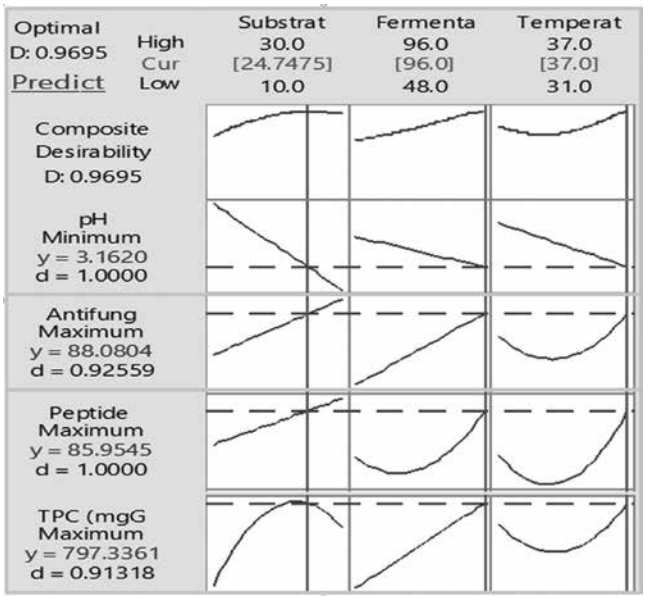

Figure 2. Response optimizer for the optimum conditions of PKC Lacto-fermentation with *L. plantarum* ATCC8014 as determined by RSM

Table 4. Experimental and fitted value of responses and *P*-values of validation for optimizing the PKC fermentation with *L. plantarum* (Four-sample t-test)

pH (Y_1)		TPC (Y_2)		Peptide content (Y_4)		Inhibition (%) (Y_5)	
EV[a]	FV[a]	EV[b]	FV[b]	EV[d]	FV[d]	EV[e]	FV[e]
3.98	4.01	715.46	714.98	58.36	59.52	65.58	68.26
3.51	3.38	759.73	737.30	73.97	67.26	77.84	75.86
4.07	3.78	748.78	747.33	66.56	67.43	75.71	77.90
3.23	3.15	760.79	769.65	75.36	75.17	79.87	85.50
4.14	4.06	747.47	741.27	65.67	61.83	75.29	75.85
3.32	3.43	756.64	763.59	67.23	69.57	78.70	83.45
3.62	3.73	742.55	749.52	72.62	69.56	71.35	79.21
3.23	3.10	766.44	771.84	77.60	77.30	81.92	86.81
3.93	3.85	743.40	755.41	64.37	67.77	70.06	74.83
3.47	3.63	801.52	787.76	80.40	75.68	89.24	84.48
3.48	3.53	768.29	763.67	76.12	75.50	82.65	78.19
3.24	3.31	805.12	796.02	82.20	83.41	89.88	87.84
3.82	3.58	759.46	761.4	63.26	61.32	77.71	76.88
3.37	3.58	757.02	761.48	61.65	61.32	76.66	76.88
3.35	3.58	754.54	761.48	59.11	61.32	74.65	76.88
3.98	4.01	715.46	714.98	58.36	59.52	65.58	68.26
3.51	3.38	759.73	737.30	73.97	67.26	77.84	75.86
P= 0.472		*P*= 0.189		*P*= 0.169		*P*= 0.828	

EV= experimental value and FV= fitted value; [a] [b] each small letters within different columns statistically not significant (P<0.05; n=3); Fitted values of response were predicted by software. F_1= C. gloeosporioides.

A differentiation was prepared of the data from the experiment with the Minitab computer software predicted fitted values to prove the accuracy of the reduced models (Table 4). It was found that the experimental response values settled with the predicted but with no significant (P>0.05) difference shown between the two values. The relative similarity of the experimental and fitted values established the accuracy of the study. Besides, the insignificant results (P> 0.05) between the predicted and experimental response values indicated that further model transformations were unnecessary. Under these fermentation conditions, the predicted models had the best antifungal ability against *C. gloeosporioides.*

Antifungal mechanism

Conidia germination of *C. gloeosporioides* was significantly (P<0.05) inhibited by both MIC and 50% MIC concentrations compared to the control. The conidia germination was reduced to 10.3 and 39.8% by the concentration with MIC and 50% MIC, respectively (Figure 3). PKCL1 treatment showed a remarkable effect in preventing the conidia germination of *C. gloeosporioides*. As the concentration of the antifungal compounds increased, a pronounced inhibition in the percentage

of conidia germination was exhibited. Several previous studies investigated the effects of different antifungal compounds on fungal conidia germination. Arulrajah *et al*. (2020) and Kwak *et al*. (2014) reported that antifungal peptides and organic acids inhibit conidia germination and induce conidial death.

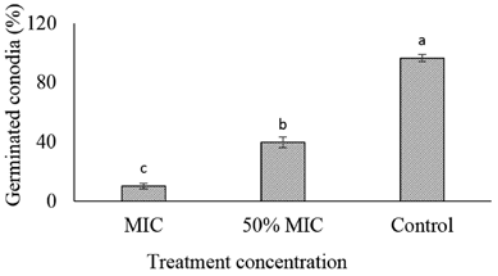

Figure 3. Effect of two concentrations of PKCL1 on percentage germinated conidia of *C. gloeosporioides*. Vertical bars indicate the standard error of the mean for three replicates. The same letters were not significantly different at *P*≤0.05. MIC= minimal inhibition concentration

Intracellular substances such as protein, sugar, and nuclein (DNA) compounds are essential for microorganisms' growth, development, and reproduction (Chen *et al*., 2018). The results showed (Table 5) that PKCL1 treatments caused higher amounts of substances leakage in *C. gloeosporioides* when compared to the control. The protein leakage in fungal mycelia was noticeably affected by the antifungal treatment. Positive control exhibited the highest protein leakage compared to the PKCL1. On the contrary, the non-treated (negative) control exhibited the lowest protein leakage.

The results showed that the total sugars and DNA leakage in fungal mycelia were significantly affected by PKCL1 compared to the negative control (Table 5). The results indicate that higher leakage of the cellular component of positive control and PKCL1 treatments, and the negative control, did not show an increment in cellular leakage. These results indicate that the positive control and PKCL1 caused the cellular leakage by damaging the cell membrane (Gomaa, 2017).

Table 5. Effect of antifungal treatments and incubation times on leakage in soluble protein, total sugars, and DNA in *C. gloeosporioides* mycelia

Factors	Soluble protein concentration (µg mL^{-1})	Total sugars concentration (µg mL^{-1})	DNA concentration (Absorbance at 260 nm)
Treatments (A)			
PKCL1	28.30^{b}	15.78^{c}	0.646^{c}
+Control	34.33^{a}	22.24^{a}	0.708^{a}
- Control	2.96^{c}	2.51^{d}	0.633^{d}

*The same letters within a column are not significantly different at P ≤ 0.05 using Turkey multiple comparison test. ** = highly significant at P ≤ 0.01. F1= C. gloeosporioides.*

Similar results have also been observed in the study of antimicrobial mechanisms with *Juniperus rigida* essential oil by Meng *et al*. (2016) . Current results revealed that PKCL1 caused protein losses , reducing sugars and DNA from the fungal mycelia, which permanently damaged the cytoplasmic membranes (Bajpai *et al*., 2013).

The results showed that PKCL1 treatments significantly (P<0.05) reduced the ergosterol biosynthesis in *C. gloeosporioides* compared to the negative control (Figure

4). Previous studies have reported that antifungal metabolites of LAB destroy the plasma membrane of the fungi and affect the membrane biosynthesis (Gómez-Guillén *et al*., 2010; Faruck *et al*., 2016).

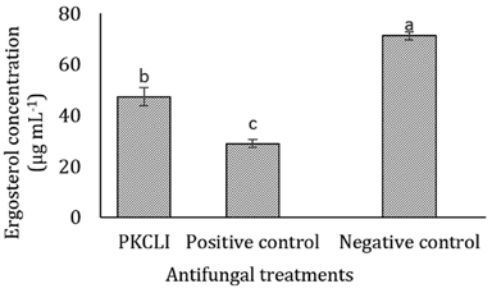

Figure 4. Effect of antifungal treatments on ergosterol concentration of *C. gloeosporioides* and *B. theobromae*. Vertical bars indicate the standard error of the mean for three replicates. The same letters were not significantly different (*P*>0.05) within the same color columns

Ergosterol is the key sterol component in the plasma membrane, and it is accountable for maintaining the primary cell function and cell integrity. Liu and Nes (2009) reported that many fungi and protozoa could not survive without ergosterol. Current results exhibited that PKCL1 reduced the ergosterol concentration in the fungal mycelia.

As shown in Figure 5, the basic mycelia structure of *C. gloeosporioides* was disrupted by the PKCL1 treatments. Thus, the cells have collapsed after the cracking, shrinkage, and swelling. In addition, some of the mycelial were enlarged, and the cell wall has been undergone partially swelling. These attributes are the antifungal effect of PKCL1, and Da *et al*., 2019 suggest that PKCL1 disturbs the biosynthesis of cell wall compounds, thereby preventing the cell wall bio synthesis. Furthermore, it was also reported that the antifungal peptides in PKCL1 alter the protein and nuclein synthesis of fungi (Faruck *et al*., 2016; Gómez-Guillén *et al*., 2010). Muhialdin *et al*. (2020b) suggested that the antifungal peptides were more capable of causing the cell wall lysis of the targeted fungi.

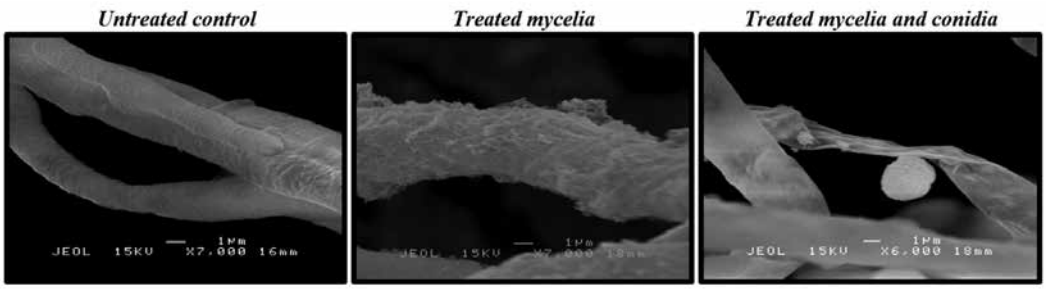

Figure 5. SEM images of PKCL1 and PKCL2 (25% MIC (5 mg mL^{-1})) treated *C. gloeosporioides* and *B. theobromae* mycelia at 25±2 °C for 4 days

Conclusions

Box Behnken design under response surface methodology was effectively applied to optimize the fermentation conditions for enhance the antifungal activity of PKCL1. The substrate ratio, fermentation time, and temperature are influenced to produce antifungal compounds. The combinations of optimum fermentation conditions were the substrate ratio of 24.75%, fermentation time of 96 hours, and fermentation temperature of 37 °C. The inhibition of *C. gloeosporioides* by PKCL1 was observed as 88.08% under invitro conditions. Significant differences were observed in the concentration of protein, DNA, sugars, and ergosterol between the treated and non-treated fungi. Thus, the PKCL1 was affected to the cell membrane structure and biosynthesis pathways of *C. gloeosporioides.* Besides, PKCL1 changed the cell morphology and destroyed the cell wall structure of *C. gloeosporioides*.

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