Optimization of the *Hermetia illucens* Larvae Extraction Process with Response Surface Modelling and Its Amino Acid Profile and Antibacterial Activity

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Optimization of the *Hermetia illucens* Larvae Extraction Process with Response Surface Modelling and Its Amino Acid Profile and Antibacterial Activity

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Abstract. *Hermetia illucens* Larvae (HiL) is a protein source that can replace soybean meal and act as functional feed in the poultry industry. This study aims to optimize the HiL extraction process and evaluate the amino acid profile and antibacterial activity. Response surface modelling (RSM) was used to optimize the HiL extraction process and yield assessment. RSM followed by three levels and three variables based on Box-Behnken design. The extraction method used alkali extraction procedure with three variables, e.g., X1: NaOH solvent, X2: HCl solvent, and X3: precipitation volume. Inhibitory zone assay used *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhimurium* and *Escherichia coli*. Model build by RSM has R^2 = 0.9949 with significant value ($P<0.01$). The correlation plot of experimental yield versus predicted yield has R^2 = 0.9974. Validation of the model has a lack of fit value ($P>0.05$) which indicate that the model is valid. The EAAI and BV value of HiL extract are 16.51 and 5.30%, respectively. HiL extract did not show antibacterial activity to inhibit gram-positive and gram-negative bacteria. The optimum yield (23.03%) was obtained by performing with NaOH 2.35 M, HCl 2.76 M, and precipitation volume 25.28 mL. EAAI of HiL extracts is slightly higher than that of dried HiL but lower than that of soybean meal.

1. Introduction

Soybean meal as the main source of protein is widely used in the Indonesian poultry feed industry because of its high nutrient content and nutritional quality. However, most of the soybean meal was imported from the Brazil and Argentina States 4,450,000.00 metric tons [1]. Therefore, very urgent to find an alternative, one of them is HiL. The nutrient composition of HiL extract was 7.05% moisture, 9.52% ash, 42.65% crude protein, 17.95% crude fat, and 6.98% crude fibre [2]. Whole HiL and extract in monogastric nutritional perspectives increase Feed Conversion Ratio (FCR) and improve performance. Based on Spranghers *et al*. [3], full-fat HiL can replace 8% of soybean in the diet without decrease the performance of a piglet. In addition, the HiL is capable as a functional feed because it has antibacterial peptides that inhibit gram-positive bacteria, e.g., *Staphylococcus aureus* and *Bacillus subtilis*. The 14th day instar 5 HiL had antibacterial peptides known as Defensin Like-Proteins (DLPrs) [4].

To increase HiL protein and extract the antibacterial peptides, a method that can be used is alkali extraction. The protein extraction method using alkali sodium hydroxide (0.18% NaOH) and precipitate with hydrochloric acid (0.1 M HCl) effectively increases rice protein to 79% higher, reported by De Souza *et al*. [5]. Alkali uses to dissolve proteins and separate them from insoluble components such as chitin, while acids are used to precipitate proteins by interfering stability of the solution. According to Park *et al*. [6], HiL extracts using distilled water, ethyl acetate, and chloroform have inhibitory activity against gram-positive bacteria *Staphylococcus aureus* KCCM 40881 and *Staphylococcus epidermidis* KCCM 35494. HiL extraction to concentrate protein and as functional feed has been widely studied, but optimization of the extraction process has not been inspected. This study aims to examine the optimization of HiL extraction processes with response surface modelling and assessment of amino acid profile and antibacterial activity.

2. Experimental details

2.1. Materials

A 14-15th days HiL was obtained from PT Sahabat Tani Farm, Bubulak Village, Bogor Regency, West Java in March 2018. HiL stored at -4 °C. HiL dried at 60 °C for 2-3 days, then ground and sieved with mesh no. 100. Gram-positive and gram-negative bacteria used include *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6051, *Salmonella typhimurium* ATCC 13311 and *Escherichia coli* ATCC 8639. *Escherichia coli* and *Staphylococcus aureus* were cultured using tryptic soy broth, while others on nutrient broth.

2.2. Methods

The steps of alkali extraction consist of alkali extraction and acid precipitation. Based on De Souza *et al*. [5] modified, HiL was dissolved using NaOH solvent (X1) 1:10 mg/mL (b/v) and homogenized 30 minutes. Then the solution was centrifuged 5000 rpm at 4 °C to produce an alkali extract (supernatant) and chitin (pellet). Alkali extracts were precipitated using HCl solvent (X2) with volume (X3) (v/v) based on the salting out mechanism, and then yield (HiL extract) was produced. The HiL extract was defusion using dialysis tube in distilled water for 30 minutes. The HiL extract was dried using an oven at 55 °C. The HiL extract based on optimal concentration and volume of X1, X2 and X3 solvents were tested for amino acid profile and inhibitory zone assay. The nutrient composition of ash, crude protein (CP), ether extract (EE), crude fibre (CF) and nitrogen-free extract (NFE) measured base on AOAC [7], except moisture according to SNI no. 01.2891 [8]. A sample of the HiL extract was sent to Laboratorium Ilmu dan Teknologi Pakan for amino acid profiling, which analyzed of the essential and non-essential amino acids. Amino acid profiles were determined using high-performance liquid chromatography and following AOAC [9] procedure. Essential amino acid index (EAAI) value calculated by Oser [10] with the equation below:

$$\log \mathrm{EAAI} = \frac{1}{10}\left[\log \frac{100 \times a1}{\mathrm{at}1} + \frac{100 \times a2}{\mathrm{at}2} + ... + \frac{100 \times \mathrm{an}}{\mathrm{at}n}\right] \quad (1)$$

note: a1 (first, ...(1, 2, 3, 4, …., n) essential amino acid from experiment sample), at1 (first, ...(1, 2, 3, 4, …., n) essential amino acid from standard (soybean meal)). Biological value solved with equation bellow [11]:

$$\text{Biological value} = 1.09(\mathrm{EAAI}) - 11.70 \quad (2)$$

Inhibitory zone assay did in PT Saraswanti Indo Genetech. The sample was dissolved with sterile distilled water 1:3 (b/v). Then, the solution is inserted into the agar well and incubated with the test bacteria at 37 °C, and the clearing zone formation is measured.

2.3. Experimental design

Three variables and three RSM levels used are NaOH concentration (X1), HCl concentration (X2) and precipitation volume of HCl (X3) using factorial Box-Behnken design (BBD). The adjusted R square (R^2) and analysis of variance (ANOVA) are used to validate the model. Data analysis did in R programming language version 3.5.2 using r-base and "library (rsm)" [12,13].

Table 1. The three levels of the three variables in the RSM assessment

Independent variables	Level		
	-1	**0**	**1**
NaOH, X1 (M)	1	2	3
HCl, X2 (M)	1	2	3
Precipitation, X3 (mL)	10	20	30

3. Results and discussion

Statistical analysis performed based on the design matrix and the corresponding results listed in Table 2. The equation model obtained with BBD and then empirical model between response and independent variables in the coded units were presented of the experimental results as follows:

$$Y = 20.452 + 3.06X1 + 3.004X2 + 3.443X3 + 3.296X1X2 + 3.561X1X3 - 2.57X2X3 - 10.626X1^2 - 1.851X2^2 - 2.599X3^2 \quad (3)$$

Where Y is the yield of HiL extract, X1, X2 and X3 are NaOH concentration (M), HCl concentration (M) and precipitation volume of HCl (mL), respectively.

Table 2. Box–Behnken design matrix and corresponding experimental and predicted response

Runs	X1 (M)	X2 (M)	X3 (mL)	Y experimental (%)	Y predicted (%)
1	3.00	2.00	10.00	3.12	3.28
2	2.00	3.00	10.00	18.36	18.13
3	2.00	1.00	30.00	18.78	19.01
4	1.00	2.00	10.00	3.45	4.29
5	1.00	3.00	20.00	5.23	4.62
6	3.00	2.00	30.00	18.13	17.29
7	2.00	1.00	10.00	7.76	6.99
8	3.00	3.00	20.00	17.27	17.34
9	1.00	1.00	20.00	5.27	5.21
10	2.00	3.00	30.00	19.10	19.88
11	3.00	1.00	20.00	4.12	4.74
12	1.00	2.00	30.00	4.22	4.05
13	2.00	2.00	20.00	20.16	20.45
14	2.00	2.00	20.00	20.75	20.45

X1: NaOH concentration (M), X2: HCl concentration (M), X3: Precipitation volume (mL)

The results of the model equation ANOVA are tabulating in Table 3. The linear coefficient for NaOH concentration, HCl concentration, and precipitation volume, significantly ($P<0.01$) affect yield (%). The interaction between NaOH concentration, HCl concentration, and precipitation volume for its combination was significant ($P<0.01$) affect the yield (%). Quadratic coefficient shows significant value ($P<0.01$), indicated response affect yield (%). The equation (3) variables sign of X2X3, X12, X22 and X32 are negative. It is mean an increasing that value decrease yield (%). On the other hand, variable X1, X2, X3, X1X2 and X1X3 has a positive relationship.

Table 3. Results for analysis of variance of the model equation

	Std. Error	t Value	P-Value	Significant
X1	0.33992	9.0016	0.0008	Sig.
X2	0.33992	8.8372	0.0009	Sig.
X3	0.33992	10.1278	0.0005	Sig.
X1 : X2	0.48072	6.8568	0.0023	Sig.
X1 : X3	0.48072	7.4085	0.0017	Sig.
X2 : X3	0.48072	-5.3460	0.0059	Sig.
$X1^2$	0.53746	-19.7710	<0.0001	Sig.
$X2^2$	0.53746	-3.4448	0.0261	Sig.
$X3^2$	0.53746	-4.8358	0.0084	Sig.

X1: NaOH concentration (M), X2: HCl concentration (M), X3: Precipitation volume (mL)

Based on Table 4, the analysis indicates that the stationary point of the fitted surface is at 2.35 M, 2.76 M and 25.28 mL within the experimental area with the optimum of theoretical yield is 23.03%. Tree eigenvalues are negative, that implies stationary point is maximum. According to Table, the comparison of yield experimental (%) versus yield prediction (%) presented in Figure 1. The correlation value is 0.9974 that mean experimental value can be described well by prediction value based on the model equation (3).

Table 4. The theoretical yield of *Hermetia illucens* larvae extract on RSM-derived optimum extraction conditions and eigenvalues

	X1	X2	X3	Theoretical yield (%)
Stationary point	2.35	2.76	25.28	23.03
Eigenvalues	-0.88	-2.82	-11.37	

X1: NaOH concentration (M), X2: HCl concentration (M), X3: Precipitation volume (mL)

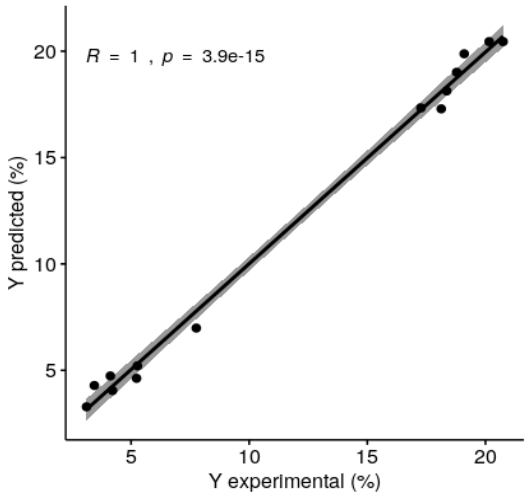

Figure 1. The plot experiments versus predicted response. Correlation value is 0.9974 with (P<0.01)

Validation of the model is described in Table 5 that was clear the second-order BBD (TWI and PQ) P-value contributed significantly to the model consequently, the canonical analysis is valid [12]. The model lack of fit F-value of 6.75 implied the model lack of fit was not significant (P>0.05) relative to pure error, as there was a 27.40% possibility that a lack of fit F-value this large could occur due to noise. The not significant of the lack of fit F-value meant the validity of the regression model. As analyzed above, this model can be used to predict the yield of HiL extract (%).

Table 5. Analysis of variance the RSM model for *Hermetia illucens* larvae extraction process

	DF	SS	MS	F-value	P-value	Significant
FO (X1, X2, X3)	3	241.90	80.634	87.2323	0.0004	Sig.
TWI (X1, X2, X3)	3	120.61	40.203	43.4935	0.0016	Sig.
PQ (X1, X2, X3)	3	362.50	120.833	130.7217	0.0001	Sig.
Residuals	4	3.70	0.924			Sig.
Lack of fit	3	3.52	1.174	6.7493	0.2740	Not-Sig.
Pure error	1	0.17	0.174			Sig.

FO: first order, TWI: two way interaction, PQ: pure quadratic

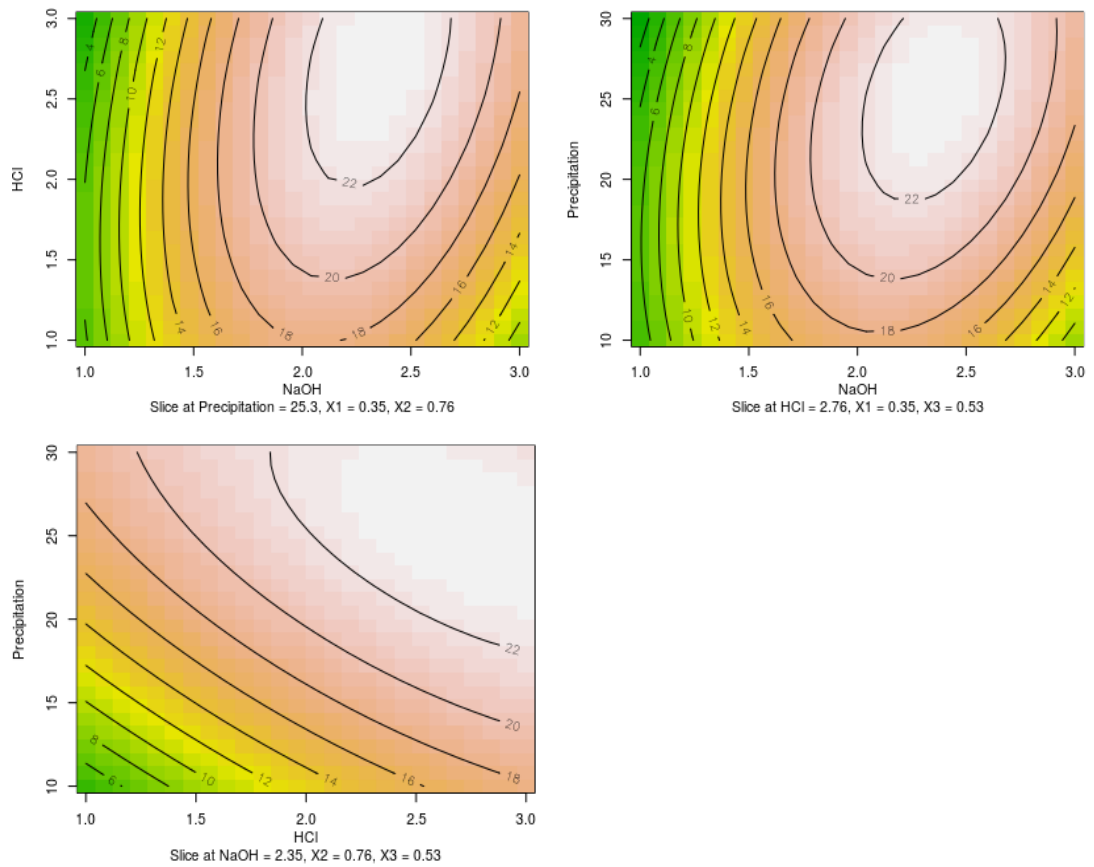

Figure 2. The counterplots of response surface modelling from *Hermetia illucens* larvae extraction process. Counterplot 1: NaOH (M) versus HCl (mL) and slice precipitation volume at 25.3 mL, counterplot 2: NaOH (M) versus precipitation volume (mL) and slice HCl at 2.75 M and counterplot 3: HCl (M) versus precipitation volume (mL) and slice NaOH at 2.35 M

Counterplots of RSM of HiL extraction process are present in Figure 2. The first counterplot NaOH concentration versus HCl concentration, an increase in NaOH concentration 2.0 to 2.5 M improved the extraction yield. However, NaOH concentration is more than 2.5 M a slight decline. An area in HCl concentration 2.0 to 2.5 M has optimal yield, with precipitation volume 20 to 30 mL. The optimal area within plot approximately at NaOH concentration more than 3.0 M combine with precipitation volume more than 30 mL.

The comparison of nutrient composition of unextracted HiL and extracted HiL listed in Table 6. Crude protein of HiL extracted increase become 33.79% compare to Dried HiL 24.98%. Ether extract

extremely decreases by 21.14%. Because of, alkali extraction process separate HiL extract becomes 3 layer parts, the bottom layer was insoluble particles (e.g. crude fibre), middle layer was solute particles (e.g. crude protein), and the top layer was ether extract. The middle layer was carried out with centrifugation and precipitate using HCl solution to get the HiL extract, so from that process the ether extract component was removed. Crude protein of HiL extract 5.51% lower than soybean meal, the alkali extraction process slightly can enhance crude protein from HiL.

Table 6. Nutrient composition of the dried *Hermetia illucens* larvae, extract and soybean

	Nutrient composition (%)					
	Moisture	Ash	CP	EE	CF	NFE
Dried HiL	7.40	10.23	24.98	22.94	4.02	30.43
HiL extract	10.81	13.24	33.79	7.56	1.80	32.80
Soybean meal[a]	12.4	7.10	39.30	2.00	6.70	20.90

CP: crude protein, EE: ether extract, CF: crude fibre, NFE: non-fat extract, a: Jayanegara [14]

Table 7 are amino acid profiles, as the standard of an essential amino acid index is soybean meal. Dried HiL and HiL extract have EAAI 14.96 and 16.51%, respectively. Extraction process increases EAAI by 1.58%. It is inherent with BV, that extraction process can increase BV by 1.70%. In nutritional view, soybean meal is the best protein source, comparing with to HiL extract. EAAI and BV can describe the nutritional value [11].

Table 7. Amino acid profile of the dried *Hermetia illucens* larvae, extract and Soybean meal

	Dried HiL	HiL extract	Soybean meal
Essential[a]			
Phenylalanine (%)	0.18	0.20	1.30
Valine (%)	0.14	0.16	1.04
Threonine (%)	0.10	0.11	1.00
Isoleucine (%)	0.14	0.16	1.06
Methionine (%)	0.13	0.14	0.75
Cysteine (%)	0.06	0.08	0.55
Histidine (%)	0.14	0.18	1.36
Arginine (%)	0.33	0.34	1.48
Leusin (%)	0.35	0.36	1.54
Lysine (%)	0.30	0.28	1.39
Non Essential[a]			
Aspartic Acid (%)	0.33	0.38	1.45
Glutamic Acid (%)	0.43	0.57	0.85
Serin (%)	0.12	0.14	0.68
Glycine (%)	0.20	0.23	1.32
Alanine (%)	0.12	0.18	1.16
Proline (%)	0.23	0.30	1.14
Tyrosine (%)	0.12	0.14	1.35
EAAI (%)	14.96	16.51	100.00
BV (%)	4.60	6.30	97.30

EAAI: essential amino acid index, BV: biological value, a: conversion base on 100% dry matter

Table 8. Inhibitory zone assay of the dried *Hermetia illucens* larvae, extract and zinc-bacitracin

	Dried HiL	HiL extract	Zinc-bacitracin
Staphylococcus aureus ATCC 6538 (mm)	ND	ND	23.17 ± 0.60
Bacillus subtilis ATCC 6051 (mm)	ND	ND	19.50 ± 1.65

Salmonella typhimurium ATCC 13311 (mm)	ND	ND	14.00 ± 0.44
Escherichia coli ATCC 8639 (mm)	ND	ND	19.07 ± 1.63

ND: not detected

Table 8 describes inhibitory zone assay, HiL extract and not extracted did not have antibacterial activity. Besides that, zinc-bacitracin has an antibacterial activity to inhibit gram-positive and gram-negative bacteria. It is mean neither HiL extract nor dried HiL cannot express antibacterial peptides or extraction procedure needs to evaluate. This result is different with Park *et al*. [15], purified HiL using HPLC and fast protein liquid chromatography present inhibitory activity against gram-negative bacteria (e.g. *Escherichia coli* KCCM 11234) 0.52-1.03 (μM). And Park *et al*. [16], purified HiL inhibit gram-positive bacteria (e.g. *Staphylococcus aureus* KCCM 12256), 10 μg/mL.

4. Conclusion

In conclusion, a new optimization method based on an RSM was investigated for the extraction of *Hermetia illucens* larvae. The maximum extraction yield was obtained by performing with NaOH 2.35 M, HCl 2.76 M, and precipitation volume 25.28 mL. Under these conditions, the predicted yield is 23.03%. EAAI for HiL extracts slightly higher than dried HiL, but lower than soybean meal. HiL extract did not present antibacterial activity.

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