An approach to optimize process parameter for peptides extraction from *Zophobas morio* (Fabricius) using antifungal activity as the response

[1*]Yusof, F., [1]Faruck, M.O. and [2]Chowdhury, S.

[1]*Department of Biotechnology Engineering, Kulliyyah of Engineering, International Islamic University Malaysia, P.O Box 10, Jalan Gombak, 50728 Kuala Lumpur, Selangor*
[2]*Department of Mechatronics Engineering, Kulliyyah of Engineering, International Islamic University Malaysia, P.O Box 10, Jalan Gombak, 50728 Kuala Lumpur, Selangor*

Abstract

Antifungal peptides have been successfully extracted from whole body larvae of *Zophobas morio* (Fabricius) by using acidified isopropanol. To ensure that the extraction is cost effective for maximum yield, Response Surface Methodology (RSM) using a Central Composite Design (CCD) strategy was adopted to optimize the extraction process parameters. The effect of independent parameters, namely, the homogenization temperature (°C), homogenization time (min) and solid (g) to the solvent (ml) ratio of the extraction process on the fungal growth was studied. The extracted samples obtained by conducting runs accorded by the experimental design showed varying degree of antifungal activity against *Aspergillus niger,* the selected fungal strain, as assayed by the ''Poisoned agar technique'. The investigation showed that the optimum values of the extraction parameters for the maximum antifungal peptides were 5 minutes homogenization time, 4°C homogenization temperature and 3.5:1 solid to solvent ratio. This study reports the development of an extraction process that allows careful recovery of antifungal peptides from larvae. In the validation of the experimental model, the error between the actual value and the predicted value was determined to be 3.57%.

Introduction

The problem of microbial resistance is increasing and the outlook for the use of existing antimicrobial drugs in future is still uncertain. A broad range of antifungal medicines are available but causes toxicity in the human body. The reckless use of antibiotics for fungal infections results in antibiotic resistance and this is a major issue related to the treatment with antibiotics. Therefore, actions must be taken to reduce the problem, one which is to continue studies in the development of new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial drug to the patient. In this regard, one of the efforts is the research and development in a new class of antimicrobial compound which has been found in peptides.

Nowadays, peptide research is prominent in lieu of its therapeutic efficacy against various pathogenic microbes. Peptides are small and cationic component of the innate immune system of numerous organisms (Faruck *et al.*, 2016a) which shows that nature has potential sources of peptides. The diversified resistance of pathogens towards available antifungal agents has necessitated the isolation of peptides from the natural sources

Several extraction processes have been carried out to extract the peptides from natural resources, such as from plants, animals, microbes and insects. In relation to insects, peptides have been extracted from larvae stage darkling beetle *(Zophobas morio)* which has shown to exhibit various bioactivities, including antifungal activity (Fu *et al.*, 2009; Mohtar *et al.*, 2014; Faruck *et al.*, 2015; Chowdhury *et al.*, 2015). Peptides extracted from *Musca domestica* (housefly) with a molecular weight of 17 KDa was reported to inhibit the growth of *Candida albicans* (Fu *et al.*, 2009). Gundappa *et al.* (2012) reported that peptide extract of dung beetle showed activity against *Candida albicans*. Mohtar *et al.* (2014) has shown that extract of *Zophobas morio* larvae exhibited antimicrobial activity against, *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Chowdhury *et al.* (2016) has shown that the same extract was able to significantly inhibit the growth of MCF-7, a cancer cell line.

In this study, antifungal peptides were extracted from whole body of *Zophobas morio* larvae or more commonly named as super mealworm. The cheaply reared larvae are mainly used commercially as animal pet feeder and previous work showed that it

*Corresponding author.
Email: yfaridah@iium.edu.my

is rich in protein (Zhang *et al*., 2011). The medium component is one of the major parameter that influences peptide activity and thus its optimization is essential to enhance peptide activity (Wang and Liu, 2008). Similarly, the process temperature is another important parameter for peptide extraction as lysis of the cells or tissues is usually achieved at low temperatures (Martínez *et al*., 2013). According to Fila *et al.* (2011), perfect homogenization for release of solute from cell tissue consists of suitable choice of extraction protocols, including the choice of component of its extraction medium. Furthermore, homogenization time is a crucial parameter in any extraction process especially for cells with tough cell wall, like insects. Insects are well-known to have tough tissue wherein the disruption of the resistant cell wall needs to be done vigorously and requires substantial time, as being recommended by Fila *et al.* (2011) in their work in protein extraction from tobacco pollen .

As the extraction process is important for maximally and efficiently extracting the targeted peptides, the process parameters used, need to be carefully considered. Application of statistical optimization in experimental studies is intended primarily to find the effective patterns of interaction of the selected parameters and reducing the number of experimental runs and this can subsequently decreased the overall time required for the experiments (Ortega *et al*., 2003; Chowdhury *et al*., 2016). For the optimization process, Response Surface Methodology (RSM) was used to design and analyse the data. This statistical technique is able to explore the relationships between several explanatory parameters and one or more response parameters. The main idea of RSM is to use a sequence of designed experiments to obtain an optimal response, thus it can be used to maximize the extraction yield, in this case, antifungal peptides, by optimizing the process parameters. Statistical analysis of data via analyses of variance (ANOVA) will enable the degree of accuracy held by the derived model to be evaluated based on the experimental responses. In this regard, scientists have used various statistical softwares, however, the Design Expert® (Version 7.0.0, Stat-Ease Inc., Minneapolis, USA) was employed in this study. The design included three important extraction process parameters, homogenization time (min), homogenization temperature (°C) and solid (g) to solvent (ml) ratio with the degree of antifungal activity against one type of fungus, *Aspergillus niger,* was used as the response. The validation experiment was conducted based on the suggested solution from the software.

Materials and methods

Source of Zophobas morio

The late instar larvae of *Zophobas morio* from the stock culture was subjected to whole body extraction. Since there is no morphological study to distinguish between larval stages, the determination of final instar was based on the body weight (Gundappa *et al*., 2012). Each larvae body weight was around 0.7 to 0.8 g. The larvae were maintained in a 1000 g substrate mixture with ground chicken and wheat bran and bubble rice (chicken bran:wheat bran:bubble rice; 2:1:1) as a food supplement. Both the insect and the food supplement were kept in a plastic container of dimension 60 cm x 50 cm x 30 cm with holes to allow ventilation and air circulation. Each plastic container accommodated 250 super mealworms with different stages. The larvae were kept in the laboratory at 25ºC to 32.5ºC with 55% to 90% Relative Humidity (RH) in a 12h:12h, Light:Day cycle. The food was changed every two weeks after cleaning and rinsing. Carrot slice was used as the source of water and changed thrice a week while carcasses of dead insects were removed every day to avoid contamination.

Extraction procedure of antifungal peptides

Non immunized late instar larvae of *Zophobas morio* from the stock culture was subjected to whole body extraction following the method of Mohtar *et al.* (2014). Only larvae with a body weight of around 0.70 g to 0.80 g were selected for the study. Initially, the larvae were frozen for half an hour to restrict their movement. Then, the larvae were washed with distilled water twice and with 70% ethanol once, to remove contaminants and dried with tissue paper to remove excess ethanol. They are kept for 1 hour in -20°C freezer to kill, followed by blending in the electric blender for 5 min with acidified (trifluoroacetic acid) isopropanol. The exoskeleton debris was removed by first filtering using a nylon cloth, followed by centrifugation at 12000 rpm for 40 min at 4°C to collect the supernatant. The supernatant was left to evaporate to dryness and dissolved in tri sodium phosphate buffer (pH 7.0). To remove any lipid in the sample, ethyl acetate and n-hexane was added and vigorously shaken (Leem *et al.*, 1996). A lipophilic layer formed on the surface was removed followed by evaporation to remove excess organic solvent yielding an aqueous clear extract. Extracts were freeze-dried and kept in -20°C until further use.

Antifungal Bioassay by 'Poisoned Agar' method

Sabouraud dextrose agar (SDA) plates were

prepared with the addition of crude sample of *Zophobas morio* larvae extract. This was done by first autoclaving the medium, after which, crude sample extract was added to the medium up to 5% (v/v) and gently mixed to achieve thorough mixing of the contents. For the control plate, no extract was added. After solidification of the medium, a 5 mm mycelial plug of *Aspergillus niger* was inoculated in the centre of the Petri plates and then incubated at room temperature for 5 days. Each test was triplicated. After the incubation period, the average diameter of fungal colonies was measured and the percentage of fungal growth inhibition was calculated as follows:

Fungal growth inhibition (%) = (Diameter of control colony – diameter of test colony)/(Diameter of control colony) x 100   (1)

Optimization of peptide extraction process

The optimization study of the extraction process aims at obtaining the best combination of various extraction parameters for maximal antifungal activity. The Design of Experiments (DOE) and the analysis of data was conducted using the Design Expert® computer software. Central Composite Design (CCD) under Response Surface Methodology (RSM) was chosen for the study. The independent variables used were homogenization time (min), homogenization temperature (°C) and solid (g) to solvent (ml) ratio, while the antifungal activity against *Aspergillus niger* (% inhibition), was selected as the response. According to the prescribed design, 17 combination runs were conducted with three replications at the center point. Statistical analysis via ANOVA was conducted to evaluate the degree of accuracy held by the derived model based on the experimental responses.

Results and discussion

Optimization of process parameters for extraction of antifungal peptides

In this study, three important parameters, in relation to the extraction of antifungal peptides from supermeal worm has been selected for CCD under RSM, and they are homogenization time in minutes (A-'Time'), homogenization temperature in °C (B-'Temperature') and solid to the solvent ratio (C-'Ratio'). Analysis of variance (ANOVA) was used to analyse and to check the adequacy of the model. Upon the analysis of the responses observed from the CCD experimental runs, significant parameters for the extraction process, as well as the degree of interaction between the parameters can be confirmed.

The CCD consisting of three variables in coded and uncoded forms along with anti-fungal activity (% inhibition) as the responses are shown in Table 1. The highest response determined was 86% fungal growth inhibition in Runs 11 and 15 followed by 85% in Run 9. Incidentally all the mentioned runs, Runs 9, 11 and 15 are from the triplicated centre values which has been suggested by the software, whereby all these runs are carried out at 5 minutes homogenization time, 4°C homogenization temperature and 3.5:1, solid (g):solvent (ml) ratio.

The ANOVA carried out using response surface methodology (RSM) warranted a model reduction due to some insignificant values. Data of analysis are presented in Table 2. Analyses showed that A, C, A^2, B^2 and C^2 are significant model terms, having p-values <0.0001. The F-test value is 97.23 with p<0.0001 implies that the model is significant. The coefficient of determination (R^2) value of 0.983148 is a good agreement with the adjusted determination coefficient (R^2Adj) value of 0.973036. The lack of fit value of 3.68 was not significant relative to pure error with "Prob>F" value 0.2310. The developed model equation in terms of coded factors is as follows:

Anti-fungalActivity=+84.83+1.00*A+0.40*B+2.90*C-4.70*A^2-4.70*B^2-4.20*C^2 (2)

In this equation, the coefficient of the factors represents the effectiveness of this particular factor, where a positive sign indicates synergetic effect and negative sign represents antagonistic effect. Based on the values of coefficients in the equation, 'Ratio' has the highest efficiency among the three parameters, however, the effect is antagonistic, while the other two parameters, 'Time' and 'Temperature', gave synergetic effect (Fila *et al.*, 2011).

The regression equation applied for the investigation of the interaction between variables and the optimum level determination of each factor for maximum peptide extraction can be graphically represented by 3D response surface plots. A 3D response surface plot usually shows the level of interaction between two variables when the other third variable is maintained at its optimum level. Figures 1(a)-(c) show the interaction plots between 'Time' and 'Temperature', 'Ratio' and 'Temperature' and 'Time' and 'Ratio' respectively, in relation to the antifungal activity. All gave somewhat elliptical contour suggesting the optimum operating conditions were well defined and the effects between each two given variables are significant. The shape obtained from the entire response surface plots indicate that the optimal condition for extracting antifungal peptides

Table 1. The CCD design in coded and uncoded values along with responses in anti-fungal activity (%).

Run	A (time, min)	B (Temperature, °C)	C (Ratio, g/ml)	Anti-fungal activity (%)
1	-1 (3)	0 (4)	0 (1:3.5)	79
2	1 (7)	1 (8)	-1 (1:2.5)	69
3	-1 (3)	-1 (0)	-1 (1:2.5)	66
4	-1 (3)	-1 (0)	1 (1:4.5)	73
5	-1 (3)	1 (8)	1 (1:4.5)	74
6	0 (5)	0 (4)	-1 (1:2.5)	78
7	1 (7)	0 (4)	0 (1:3.5)	80
8	-1 (3)	1 (8)	-1 (1:2.5)	68
9	0 (5)	0 (4)	0 (1:3.5)	85
10	1 (7)	-1 (0)	-1 (1:2.5)	70
11	0 (5)	0 (4)	0 (1:3.5)	86
12	0 (5)	1 (8)	0 (1:3.5)	80
13	1 (7)	-1(0)	1 (1:4.5)	75
14	0 (5)	0 (4)	1 (1:4.5)	82
15	0 (5)	0 (4)	0 (1:3.5)	86
16	1 (7)	1 (8)	1 (1:4.5)	76
17	0 (5)	-1 (0)	0 (1:3.5)	79

Table 2. Analysis of variance (ANOVA) for extraction process using the antifungal activity as the response.

Source	Sum of squares	DF	Mean square	F Value	p-value Prob>F	Status
Model	611.9804	6	101.9967412	97.2310503	< 0.0001	significant
A-Time	10	1	10	9.53276047	0.0115	
B-Temperature	1.6	1	1.6	1.52524168	0.2451	
C-Ratio	84.1	1	84.1	80.1705156	< 0.0001	
A^2	59.29099	1	59.29099123	56.5206818	< 0.0001	
B^2	59.29099	1	59.29099123	56.5206818	< 0.0001	
C^2	47.35703	1	47.35702897	45.1443214	< 0.0001	
Residual	10.49014	10	1.049014085			
Lack of Fit	9.823474	8	1.227934272	3.68380282	0.2310	not significant
Pure Error	0.666667	2	0.333333333			
Cor Total	622.4706	16				
R-Squared	0.983148					
Adj R-Squared	0.973036					
Pred R-Squared	0.947159					
Adeq Precision	27.25494					
Std. Dev.	1.024214					

from *Zophobas morio* depends on all the three variables tested. In addition, the interactions between variables can also be clearly seen from perturbation plots of Figure 1(d) which shows rather notable interaction between each set of variables. Thus, based on the statistical analysis and perturbation plot analysis, the optimum extraction condition for antifungal peptides is 5 minutes homogenization time, 4°C homogenization temperature and 3.5:1 solid to solvent ratio. The 'Ratio' is the most protuberant variable of the model as observed from the above equation. In the validation of the experimental model, the average error between the actual and the predicted values was determined to be 3.54%.

In this study, antifungal effect were carried out against a selected fungus, that was, *Aspergillus niger*. Previously, experimental runs using On-Factor-at-a-Time (OFAT) design on effect of extraction process parameters has been carried out involving four fungi namely, *Aspergillus niger, Microsporum canis, Candida albicans* and *Blastomyces dermatitids* (Faruck *et al.*, 2016b). Based on the quantitative statistical analysis, the degree of potency towards these four fungi showed almost similar values and similar trends, and the statistical differences of extraction process parameters among the four fungi tested are not significant (p>0.5). Thus here, in this study, in acquiring the optimized process parameters for maximal extraction by RSM, *Aspergillus niger* has been randomly chosen based on the laboratory compatibility, availability and easy analysis on the morphological changes. It can be inferred that the same RSM results may be observed if other three fungi strains were used.

Experimental validation

To verify the optimization results and validate the developed model, an experiment was performed
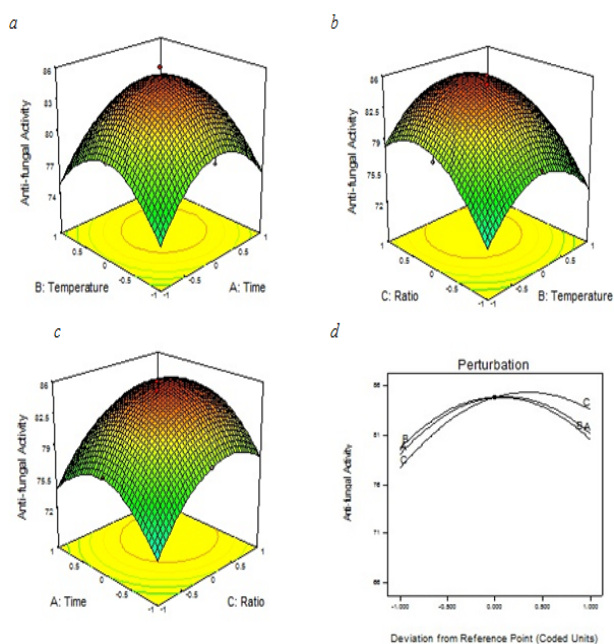
Figure 1. A 3D surface response plot of extraction process of antifungal peptides from whole body *Zophobas morio,* (a) interaction between 'Time' and 'Temperature', (b) interaction between 'Ratio' and 'Temperature' and (c) interaction between 'Time' and 'Ratio' (d) Perturbation plot.

according to the optimum production conditions of peptide extraction process. The optimum values of each experiment element in coded units were as follows: A= -0.7746, B= -0.3048 and C= -0.673, which actually demonstrate homogenizing time of 3.45 minutes, 2.78°C solvent temperature and 1:2.83 solid:solvent ratio and according to the model equation, the predicted growth inhibition was 76.81%. Upon carrying out the experimental verification process, which was done in triplicates, the antifungal growth inhibition was observed to be 73.00%, 75.00% and 74.20%, giving a mean value of 74.07% ± 1.4% which was quite close to the predicted response of 76.81%. The error between the actual value and the predicted value was determined to be 3.57%.These results therefore, verified the predicted values and revealed the efficiency of the model, signifying the optimized extraction condition for peptide production.

Conclusion

Statistically based investigational technique is confirmed to be a valuable tool in optimizing the extraction process of antifungal peptides from whole body larvae stage *Zophobas morio*. Moreover, Central Composite Design (CCD) under Response Surface Methodology (RSM) effectively determined the optimum levels of the factors that significantly

influenced the extraction of antifungal peptides. The optimum values of the extraction parameters for antifungal peptides from the whole body of supermeal worm are 5 minutes homogenization time, 4°C homogenization temperature and 3.5:1 solid to solvent ratio. The crude extract was able to demonstrate the maximum antifungal activity of 86% inhibition towards the growth *Aspergillus niger*. Statistics showed that ratio of solid to solvent is the most significant variables, however, it affects the process antagonistically. Although much work in this area has been done with *Musca domestica* (housefly) insect, this is the first time where *Zophobas morio* Fabricius, was used. The results obtained clearly suggest that the extracted crude sample could be used effectively as an antifungal agent for the treatment of several fungal diseases in human. Lastly, further studies comprising purification and characterization of the constitutive antifungal peptides in the crude sample extracted from *Zophobas morio* may lead to the discovery of new classes of antifungal peptides.

Conflict of Interest

Authors don't have any conflict of interest.

Acknowledgments

References

Chowdhury, S., Yusof, F., Faruck, M.O. and Sulaiman, N. 2015. Investigation of anticancer peptides from *Zophobas moria* Fabricus. The 12th Asian Congress on Biotechnology, Malaysia, November 15-19, 2015. Malaysia

Chowdhury, S., Yusof, F., Faruck, M.O. and Sulaiman, N. 2016. Process optimization of silver nanoparticle synthesis using Response Surface Methodology. Procedia Engineering 148: 992-999.

Faruck, M.O., Yusof, F. and Chowdhury, S. 2015. Recovery of antifungal peptides from *Zophobas morio* Fabricus. The 12th Asian Congress on Biotechnology, Malaysia, November 15-19, 2015. Malaysia.

Faruck, M.O., Yusof, F. and Chowdhury, S. 2016a. An overview of antifungal peptides derived from insect. Peptides 80: 80-88.

Faruck, M.O., Yusof, F. and Chowdhury, S. 2016b. Effect of some parameters on the extraction process of antifungal peptides from Supermeal worm, *Zophobas morio* (Fabricius), Proceedings of International Conference on Biotechnology Engineering (ICBioE

2016), 162-165, Kuala Lumpur, 25-27 July 2016 (ID:66), ISBN: 978-983-42978-8-6.

Fíla, J., Čapková, V., Feciková, J. and Honys, D. 2011. Impact of homogenization and protein extraction conditions on the obtained tobacco pollen proteomic patterns. Biologia Plantarum 55(3): 499-506.

Fu, P., Wu, J. and Guo, G. 2009. Purification and molecular identification of an antifungal peptide from the hemolymph of *Musca domestica* (housefly). Cellular and Molecular Immunology 6(4): 245-251.

Gundappa, S., Jayappa, J. and Chandrashekara, K. 2012. Bioprospecting for antimicrobial peptides from insects: *In vitro* antimicrobial activity of acidified methanol extract of dung beetles. Journal of Entomological Research 36(1): 41-44.

Leem, J. Y., Nishimura, C., Kurata, S., Shimada, I., Kobayashi, A. and Natori, S. 1996. Purification and characterization of N-β-alanyl-5-S-glutathionyl-3, 4-dihydroxyphenylalanine, a novel antibacterial substance of Sarcophaga peregrina (flesh fly). Journal of Biological Chemistry 271(23): 13573-13577.

Martínez-Maqueda, D., Hernández-Ledesma, B., Amigo, L., Miralles, B. and Gómez-Ruiz, J. Á. 2013. Extraction/fractionation techniques for proteins and peptides and protein digestion, p. 21-50. In Toldra, F. and Nollet, L.M.L. (Ed.) Proteomics in Foods: Principles and Application. US: Springer.

Mohtar, J.A., Yusof, F. and Ali, N.M.H. 2014. Screening of novel acidified solvents for maximal antimicrobial peptide extraction from *Zophobas moriofabricius*. Advances in Environmental Biology 8(3) Special 2014: 803-809.

Ortega, N., Albillos, S. and Busto, M. 2003. Application of factorial design and response surface methodology to the analysis of bovine caseins by capillary zone electrophoresis. Food Control 14(5): 307-315.

Wang, Z.W. and Liu, X.L. 2008. Medium optimization for antifungal active substances production from a newly isolated *Paenibacillus* sp. using response surface methodology. Bioresource Technology 99(17): 8245-8251.

Zhang, J. X, Guo, Q. and Liu, N. 2011. Isolation, purification and antioxidant activity evaluation of water soluble protein from giant mealworm beetle *(Zophobas morio)* Larvae [J]. Food Science 18: 006.