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Effect of extraction process parameters on antifungal peptides from Supermeal worm, *Zophobas morio* (Fabricius)

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Abstract

Acidified isopropanol extract of whole body larvae of *Zophobas morio* (Fabricius), which contains peptides, has been shown to exhibit an inhibitory effect towards fungal growth. The larvae, commonly known as supermeal worm are cheap and easily maintained. To make the extraction even more cost effective, it is pertinent to maximize the extraction yield and to optimize the extraction process. The aim of this study is to use the One-Factor-At-a-Time (OFAT) strategy to determine the maximum values of the process parameters for the extraction of antifungal peptides, where these values can later be used in the experimental design to optimize the extraction process. Based on importance, three parameters were selected, namely, initial homogenization temperature, homogenization time and solid (g) to solvent (ml) ratio. Maximum inhibition to fungal growth was found when the extraction was carried out as follows; using initial homogenization temperature of 4°C, homogenization time of 5 minutes and a solid (g) to solvent (ml) ratio of 3.5:1. The peptide extract displayed different degree of antifungal effect towards four selected fungi, *Aspergillus niger, Microsporum canis, Candida albicans* and *Blastomyces dermatitidsis*.

Introduction

Peptides are short chains of amino acids. Nowadays, peptides research is prominent in lieu of their important bioactivities; one of them is the therapeutic efficacy against various pathogenic microbes. Recovery of peptides from natural sources, such as from insects, and their potency against fungi has been reported, for example, a 17 kDa peptide extracted from *Musca domestica* (housefly) are able to inhibit the growth of *Candida albicans* (Fu *et al.*, 2009). Mohtar *et al.* (2014) reported that a small molecule compounds, which maybe peptides, extracted from *Zophobas morio* has shown to exhibit antimicrobial activity. Moreover, Chowdhury *et al.* (2016) has reported that the same extract was able to significantly inhibit the growth of MCF-7, a cancer cell line.

However, in the preparation of these peptidic extract, the process parameters are important for maximally and efficiently extracting peptides and thus need to be carefully considered. Solvent is one of the major parameters that influences peptide recovery (Wang and Liu, 2008). Similarly, temperature of the solvent is another important parameter for peptide extraction as lysis of the cells or tissues is usually achieved at low temperatures (Martínez *et al.*, 2013). In addition, homogenization methods and homogenization period are also crucial, especially in recovering peptides from insects which have tough tissues wherein the disruption of resistant cell wall needs to be done vigorously and timely (Fíla *et al.*, 2011).

The aim of this research is to extract antifungal peptides from whole body, larval stage, *Zophobas moria* by using acidified isopropanol. The effect of various parameters on extraction process, namely homogenization temperature, homogenization time and solid (g) to solvent (ml) ratio was studied using the traditional One-Factor-At-a-Time (OFAT) experimental design. In OFAT experiments, one parameter factor is varied at a time while keeping the others fixed at the optimal value because by using this strategy, it requires fewer resources (experiences, time, equipment, etc.) for the amount of information obtained. This can be of major importance in industries, where experiments can be very expensive and time consuming. For example, to execute full and fractional factorial designs, all observations are used to estimate the effect of each factor and each interaction, while generally only two observations in a OFAT experience is used to estimate the effect of each factor (Czitrom, 1999). The OFAT experimental runs are important to determine the optimal range of each

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selected process parameters, whereby this optimal range of values can be employed in the optimization experimental design in the later stage. In this study, crude peptides extract was tested against four fungi; *Aspergillus niger, Candida albicans, Microsporum canis* and *Blastomyces dermatitidis*.

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 was 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 measuring 60 cm x 50 cm x 30 cm with holes to allow ventilation and air circulation. Each plastic container accommodated 250 supermeal worms, at different stages. The larvae was kept in the laboratory at 25ºC to 32.5ºC with 55% to 90% Relative Humidity (RH) in a 12 h:12 h, Light:Day cycle. The food was changed every two weeks after cleaning and rinsing. Carrot slices were used as the source of water and changed thrice a week while carcasses of dead insects were removed every day to avoid contamination.

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 used. After solidification of medium, a 5 mm mycelial plug of fungus (*Aspergillus niger, Candida albicans, Microsporum canis* and *Blastomyces dermatitidis*) 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)

Extraction of peptides

Initially the larvae were frozen for half an hour to detain their movement. Then the larvae were washed twice with distilled water and then once with 70% ethanol, dried with tissue paper to remove excess ethanol and kept at -20°C for one hour to kill them. The larvae was subjected to homogenization, according to the selected time period and temperature, and at the selected solid to solvent ratio in acidified (trifluroacetic acid, TFA) isopropanol in electric blender. The homogenized mixture was filtered through the nylon cloth to remove the exoskeleton debris, then centrifuged at 12000 rpm for 40 min at 4°C, to collect the supernatant. The supernatant was evaporated to dryness and the resultant residue was dissolved in tri sodium phosphate buffer (pH 7.0). To remove the lipid, 5 ml each of ethyl acetate and n-hexane were added, vigorously shaken and the cloudy upper layer, discarded (Leem *et al.*, 1996). The sample was evaporated to dryness to remove the organic solvent and the aqueous extract was kept at -20° C for further use.

Design of experiments by OFAT

Three process parameters were selected for OFAT studies, namely homogenization temperature, homogenization time and solid (g) to solvent (ml) ratio. For homogenization temperature, the temperatures tested were 0, 2, 4, 6, 8, 12.5, 25, 37.5 and 50°C, setting the homogenization time to 3.5 minutes and the solid to solvent ratio of 3:1. For homogenization time, the time tested were 1 to 8 minutes, increasing one minute each time, setting the homogenization temperature at 4ºC and the solid to solvent ratio of 3:1, and finally for the solid to solvent ratio, the following were selected, namely, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6 and 7 g solid to 1 ml solvent, setting the homogenization time at 3.5 minutes and the homogenization temperature at 4ºC.

Results

Effect of homogenization temperature for peptide extraction process

Figure 1 shows the effect of homogenization temperature toward the extraction of peptides, using antifungal growth (% inhibition) as the response. The temperatures tested were 0, 2, 4, 6, 8, 12.5, 25, 37.5 and 50°C, setting the homogenization time to 3.5 minutes and the solid to solvent ratio of 3:1. The results are given as mean ± standard deviation of antifungal activity performed in triplicates. It is also being determined that, that the extraction yield in antifungal peptides was significantly ($p<0.05$)

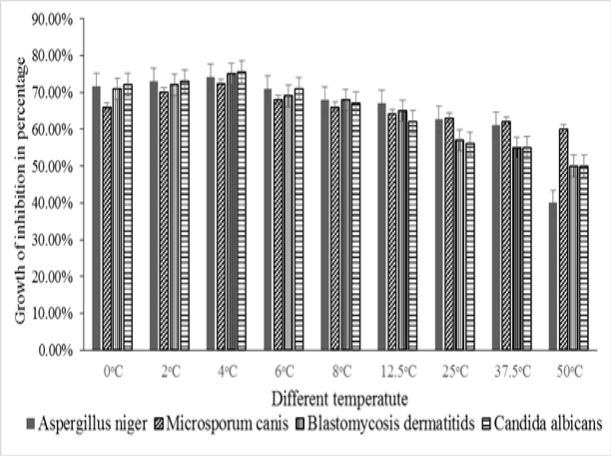

Figure 1. Effect of homogenization temperature towards peptide extraction process using antifungal growth (% inhibition) as the response. The responses were given as mean ± standard deviation, performed in triplicates. Antifungal peptides was significantly ($p<0.05$) affected by the homogenization temperature but the differences in effect of homogenization temperature among the four fungi showed non-significant differences at p value 0.991 ($p>0.05$).

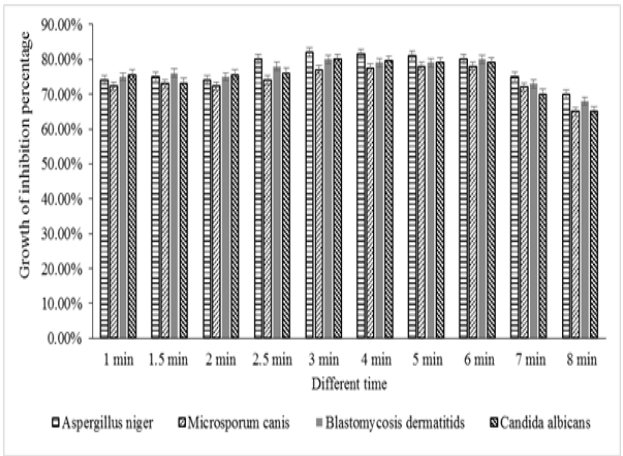

Figure 2. Effect of homogenization time towards peptide extraction process using antifungal growth (% inhibition) as the response. The responses were given as mean ± standard deviation, performed in triplicates. Antifungal peptides was significantly ($p<0.05$) affected by the homogenization time but the differences in effect of homogenization time among the four fungi showed non-significant differences at p value 0.328 ($p>0.05$).

affected by the homogenization temperature. However, the differences in effect of homogenization temperature among the four fungi (*Aspergillus niger, Candida albicans, Microsporum canis* and *Blastomycis dermatitidis*) showed non-significant differences at p value 0.991 ($p > 0.05$), thus giving similar trend of responses for all.

The results showed that there is a slight increase of activity from 0°C to 4°C, which peaked at 4°C, but increasing the homogenizing temperature higher than 4°C is of no advantage. Homogenizing the samples at 50°C, showed the least activity. Although in general, the extraction process at high temperature will improve the solubility of all solutes to ease extraction, it seems that in our study, homogenization at higher temperatures gave poor results in terms of effect on fungal growth inhibition. At this stage, the reason why it is best carried out at low temperature is still unclear. According to some researchers, cell or tissues can be lysed or broken down effectively at low temperatures to release the solute of interest and this phenomena has been shown in many studies such as for leaves (Wang *et al*., 2003), fruits (Song *et al.*, 2006), and seeds (Liang *et al*., 2006). Cell disruption method by first freezing the sample followed by blending has also been used successfully as an extraction method for some proteins (Vincent *et al*., 2006).

Effect of homogenization time for peptide extraction process

Figure 2 shows the effect of homogenization time toward the extraction of peptides, using antifungal growth (% inhibition) as the response. The homogenization time tested were 1, 2, 3, 4, 5, 6, 7 and 8 minutes, setting the homogenization temperature at 4ºC and the solid to solvent ratio of 3:1 The results are given as mean ± standard deviation of antifungal activity performed in triplicates. It is also being determined that, that the extraction yield in antifungal peptides was significantly ($p<0.05$) affected by the homogenization time. However, the differences in effect of homogenization time among the four fungi (*Aspergillus niger, Candida albicans, Microsporum canis* and *Blastomycis dermatitidis*) showed non-significant differences at p value 0.328 ($p > 0.05$), thus giving similar trend of responses for all.

The results showed that there is not much different in effect for the time range tested, as they only varies from 70% to 80% inhibition of fungi growth. A slight increase in the response was observed from 1 to 5 minutes, and it peaked at 5 minutes, however the response decreased thereon and is lowest at 8 minutes. It is very vital to provide an appropriate length of homogenizing time before peptides are released from the lysed cell wall. Previous studies has shown proof that in low molecular weight molecules extraction processes, short time homogenization was not effective enough to breakdown the cell wall to release the solute of interest (Fíla *et al.*, 2011). In this study, results showed that prolonging the homogenization time was of no advantage, as the yield dipped sharply as the time was lengthened to 7 and 8 minutes respectively. The reason of this

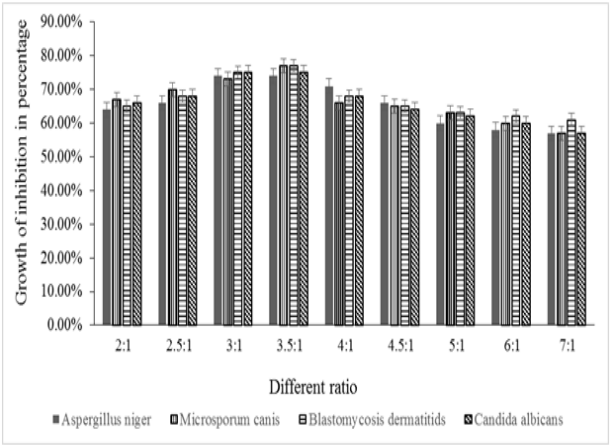

Figure 3. Effect of solid to solvent ratio for peptide extraction process using antifungal growth (% inhibition) as the response. The responses were given as mean ± standard deviation, performed in triplicates. Antifungal peptides was significantly ($p<0.05$) affected by the homogenization time but the differences in effect of homogenization time among the four fungi showed non-significant differences at p value 0.959 ($p>0.05$).

observation is still unclear.

Effect of solid to solvent ratio for peptide extraction process

Suitable ratio of solid to solvent in the homogenization mixture is very important in getting the maximum release of peptides from larvae, which will lead to the maximum effect in the inhibition of fungal gowth. The solid to solvent ratio system in the antifungal peptide extraction of insect whole body is highly advantageous from the view point of biotechnology engineering. This is because the ratio between solid to solvent can be likely optimized to increase the mass recovery rates of peptides for scale-up purposes. Solid to solvent ratio need to be carefully considered for sufficient solubility of solute of interest. In this study, the solid to solvent ratio tested were 2, 2.5, 3, 3.5, 4, 4.5, 5, 6 and 7 g solid to 1 ml solvent (acidified isopropanol) and the results showed that the recovery of antifungal peptides increased from 2:1 to 3.5:1, peaked at 3.5:1, however decreased as the ratio of solid:solvent is increased from 3.5: 1 to 7:1 (see Figure 3). According to Xi (2009) who worked with extraction of caffeine from green tea leaves, dissolution of bioactive components into the solvent is a physical process. Thus, as being observed in in this study, the first part (ratio 2:1 to 3.5:1), when the amount of larvae increases, the chance of antifungal peptides coming in contact with the solvent goes up, which leades to higher leaching-out rates. On the other hand, for solid:solvent ratio ranging between 3.5 to 7 g, the recovered peptides decreased and this may be basically due to the saturation phenomenon. In fact when the solvent is saturated with peptide components, the cellular phenomenon of diffusion stops and there exist a stabilization rate and sometimes a decrease in rate of the extracted compounds. Results also showed the trend of effect of solid to solvent ratio towards antifungal activity is similar regardless of fungi (*Aspergillus niger, Candida albicans, Microsporum canis* and *Blastomycis dermatitidis*) tested. It is also being determined that, that the extraction yield in antifungal peptides was significantly ($p<0.05$) affected by the solid to solvent ratio. However, the differences on the effect of ratio of solid to solvent among the four fungi showed non-significant differences at p value 0.959 ($p > 0.05$).

Conclusion

One-factor-at-a-time (OFAT) study has been carried out for three important parameters in peptides extraction from *Zophobas morio* larvae. The three parameters were selected based on importance as accorded by previous studies, namely, homogenizing temperature, homogenizing time and solid to solvent ratio. The results showed a significant percentage of growth inhibition against all tested fungi by the crude acidified isopropanolic extract. However, the trend of effects of all parameters tested did not vary much among the fungi tested. The highest activity was obtained when the extraction was carried out at the following conditions; 4°C for homogenizing temperature, 5 minutes for homogenization time and 3.5 g mass to 1 ml, solid to solvent ratio. These results are important, to be used further in the design of experiments to optimize the extraction process via Response Surface Methodology for maximum yield in peptide recovery.

Conflict of Interest

Authors don't have any conflict of interest.

Acknowledgments

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