# Application of Crude Protease from Cheap and Local Raw Material as a Biopesticide for the Disruption of *Pomacea Canaliculata* Eggs

Noor Hasyierah Mohd Salleh and Dachyar Arbain

***Abstract*—The application of crude protease of A*spergillus oryzae* to suppress the hatchability of *Pomacea canaliculata* eggs is highlighted. The enzyme was produced through solid state fermentation (SSF) using cassava, which is cheap and abundantly available. It was found that the enzyme suppressed the hatchability of *P. canaliculata* eggs as much as 30% and 20% at 20.8U/ml enzyme for 3 and 9 days old eggs respectively. The ANOVA result revealed that the hatchability was significantly affected by both protease concentration as well as egg's age. The $LC_{50}$ for 3 and 9 days old eggs were 3.29 U/ml and 3.54 U/ml, respectively; while $LC_{95}$ for 3 and 9 days old eggs were 19.11 U/ml and 20.09 U/ml respectively. It is proposed that the crude protease propagated from SSF using locally available and cheap material should be considered as an alternative biopesticide to suppress the hatchability of *P. canaliculata* eggs.**

***Index Terms*—*Aspergillus oryzae*, *pomacea canaliculata*, hatchability, eggs.**

## I. Introduction

The application of an enzyme as a biopesticide against several pests has been reported previously [1]. It offers advantages over chemical pesticides, even though its action may not be as fast as chemical pesticides. Ideally, enzyme-based biopesticide should be less toxic to the user and non-targeting organisms. Additionally, it is preferable to have a biopesticide which can be produced locally using cheap local raw materials [2]. Protease, a commercial enzyme belongs to many types of fungus and bacteria theoretically could cleaves the protein and disrupted the main function of protein. *Bacillus* and *Aspergillus* are among bacteria and fungus species that are well known to excrete protease [3]-[6]. *Aspergillus* is widely known for its capability to grow on solid substrate for its physical support and as a source of nutrient [7], [8]. For enzyme-based biopesticide preparation, solid state fermentation (SSF) is preferable over submerged fermentation (SMF) because it does not require sophisticated equipment such as fermenter and it can utilize cheap and locally available raw materials.

*Pomacea canaliculata* is a freshwater snail that has become a potent pest in paddy field due to its feeding habits towards young stem and leaves of paddy [9]. The vast *P. canaliculata* invasion has prompted researchers and farmers to seek for proper and effective control of this snail [10]. Most of the approaches are aimed at controlling the snail's flesh. However, even though controlling the flesh is important, it is equally important to suppress the hatching of *P. canaliculata* eggs [11]-[14]. It is known that the eggshell generally covered by cuticle layer which consists of >85% protein [15] and served as a protection against dehydration and bacterial penetration onto the eggshell [16]-[19]. The application of a commercially available protease has initiated the disruption of this cuticle, thus resulted in the suppression of the hatchability of *P. canaliculata* eggs, has been previously described [20], [21]. In this case, protease might denatured the protein within the cuticle, hence exposed the eggs to harsh environment which consequently affected its hatcability. Despite its efficacy, the use of commercial protease as a biopesticide to suppress the hatchability of *P.canaliculata* could be too expensive for the farmers, particularly in the developing countries where the invasion of the snail prevails. Therefore, it is deemed necessary to find an alternative source of protease enzyme preferably using cheap and locally available materials through simple propagation method.

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## II. Materials and Method

### A. Pomacea Canaliculata Egg Collection

The fresh eggs of the snails were collected from a nearby riverbank area of paddy field in Perlis, Malaysia and were kept in an open breeding chamber (62 cm × 40 cm × 46 cm), which was filled with tap water to provide humid condition as was described previously [22] with a slight modification. After 3 and 9 days, the eggs were located in separate containers and the mass of each egg was 0.5 g for the hatchability studies.

### B. Aspergillus Oryzae Propagation

*Aspergillus oryzae* was propagated through solid state fermentation method, using tapioca as the carbon source based on the previous report [23] with a slight modification. Briefly, the peeled tapioca was washed thoroughly, and ground before drying overnight in an oven at 50 $^{o}$C. 2 g of the prepared tapioca was placed in a shake flask and mixed well with 50 ml of distilled water and 10 ml of 10% glucose before autoclaving altogether at the 121 OC for 15 mins. These were prepared in triplicates. $2 \times 10^{6}$ cfu/ml of *A. oryzae* was added into the flask aseptically before incubating at 37 $^{o}$C. Samples which were harvested every 24 hrs were added with 50 ml distilled water before vortexing for 5 mins. The samples were

then centrifuged at 8000 rpm for 5 mins to separate between the precipitates and the supernatant fluid. The supernatant were then kept for protease assay at 4 $^{o}$C while the precipitates were dried overnight at 90 $^{o}$C to measure the cell dry weight.

### C. Bacillus sp. Propagations

*Bacillus subtilis* media was used to propagate as previously described [24]. Quantitatively (g/l), glucose, 2, casein 0.5, pepton, 0.5, yeast extract 0.5, and 50 ml salt solution (g/l) $KH_2PO_4$ 5, $MgSO_4.7H_2O$ 5, and $FeSO_4.7H_2O$ 0.1, pH 7.0 were prepared. The media was autoclaved at 121 $^{o}$C for 15 mins.

The media for *Bacillus thuringiensis* was propagated as reported previously [25]. Briefly, the media contained (g/l): $MgSO_4.7H_2O$ 0.5, $MnSO_4.7H_2O$ 0.1, $FeSO_4.7H_2O$ 0.001, $CuSO_4.5H_2O$ 0.0005, $ZnSO_4.7H_2O$ 0.0005, $CaCl_2$ 0.1, glucose 10 and pepton 5. Pepton was dissolved in phosphate buffer 0.05M, pH 7.2. $CaCl_2$ and glucose was autoclaved separately. The media was autoclaved 121 $^{o}$C for 15 mins.

Inoculum of *B. thuringiensis* and *B. subtilis* (48 hr old culture) were prepared in nutrient broth and 10 % of the inoculum were added to the sterilized growth media for protease production. Both of the growth media were agitated at 180 rpm at 37 $^{o}$C. Each sample was prepared in triplicate. Sampling was performed every 60 mins during the lag phase and every 30 mins during the log and stationary phases. Turbidity of the samples was measured at 600 nm and the protease produced was assayed respectively.

### D. Protease Assay

Protease was assayed spectrophotometrically according to K.R. Sugumaran, *et al*. (2012) [26]. Casein (0.6 %) was used as the substrate. 1 ml of casein was mixed with 200 µl of both filtrates before being incubated for 30 min at 37$^{o}$C. The reaction was stopped with the addition of 1 ml of trichloroacetic acid (0.4 mol/l) for 30 min prior to centrifugation to separate the precipitate. 1 ml of the filtrate was mixed with 2.5 ml of $Na_2CO_3$ (0.6 mol/l) and 0.5 ml of Folin Ciocalteu and was incubated for 30 min before measuring the absorbance at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute.

### E. Hatchabilities Studies

From the three types of microbes studied, only one particular microbe was selected for hatchability studies. This was attributed by the maximum production of protease. The protease therefore was prepared in different concentration from 1.027 U/ml to 20.8 U/ml. The effect of two factors, namely the protease concentration and the egg's age on the hatchability, were studied. As a comparison of the crude protease effect on *P. canaliculata* egg hatchability, commercial protease was used as a control. Protease of *Aspergillus oryzae* was purchased from Sigma Aldrich (Kuala Lumpur, Malaysia) and was prepared in different concentrations (0.5 – 4 U/ml). 1 ml of the enzyme solution of either crude or commercial protease was introduced to 0.4 g of *P. canaliculata* eggs in five replicates. The protease was rinsed a few times onto eggs to ensure it covered the eggs. The treated eggs were then placed in the breeding chamber (62 cm × 40 cm × 46 cm) which was previously filled with tap water to provide a humid condition. Their hatchabilities were observed and recorded after 14 days.

## III. Results and Discussion

### A. Protease Production from Three Types of Microbes

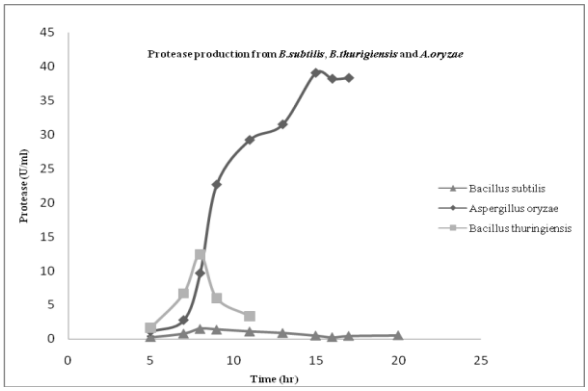


Fig. 1. Protease production from *B. subtilis*, *B. thuringiensis* and *A. oryzae.*

The crude protease production from three types of microbes were assayed (Fig. 1). It was found that *Aspergillus oryzae* produced relatively higher activity of crude protease (39U/ml) compared to *Bacillus subtilis* and *Bacillus thuringiensis*. In fact, the crude protease produced by *A. oryzae* is four times higher than *B. thuringiensis*. Hence, it showed that tapioca which is a local and cheap raw material is a good carbon source to initiate the *A. oryzae* growth and enhanced the protease production. In this case, it also showed that SSF can produce protease higher than SMF.

### B. Effect of Crude Protease Treatment on the Hatchability of 3 (Three) and 9 (Nine) Days Old of P. Canaliculata Eggs

Based on the result from Fig. 1, the crude protease obtained from tapioca which is a cheap and locally available raw material was used to suppress the hatching of 3 (three) and 9 (nine) days old of *P. canaliculata* eggs. The result is as shown in Fig. 2.

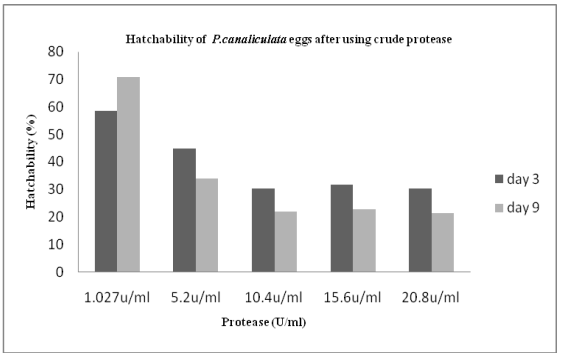


Fig. 2. Effect of crude protease treatment on the hatchability of 3 (three) and 9 (nine) days old of *P.canaliculata* eggs.

The hatchability of both 3 and 9 days old eggs was reduced when the protease concentration was increased. The hatchability for 9 days old eggs were lower compared to 3 days old eggs for all crude concentration of protease except for the concentration of 1.027U/ml. At this low crude protease concentration, the hatchability for 9 days old eggs

were higher than that for 3 days old eggs. This might be due to the fact that the embryo of 9 days old eggs had almost completely developed as indicated by the red-fade color of its shell. In comparison, the color for 3 days old eggs was still reddish.

When the concentrations of crude protease were more than 5.2U/ml, the hatchability for 3 days old eggs were reduced from 44% to approximately 30%. A similar observation was recorded in the same range of crude protease concentration, for the 9 days old eggs, where the hatchability was reduced from 34% to 20%. In this condition, the cuticle and the eggshell of 9 days old eggs were getting thinner preparing for the hatching process which normally occurred on day 12. Therefore, the high crude protease concentration had efficiently coagulated on the thinning cuticle and well absorbed into the eggshell, thus affected the hatchability of the eggs. It was believed that the *A. oryzae* also released other enzymes apart from protease such as amylase [23], [27] which therefore reduced the hatchability.

This finding was in a good agreement with a previous report which found that the growth of *Paecilomycis lilacinus* on the *P.canaliculata* eggs has successfully stopped the hatching process and the responsible enzyme were identified as lipase, protease, chitinase and amylase [14].

### C. ANOVA Studies

The ANOVA studies were carried out to investigate the significant factors involved in the hatchability treatment (Table I). It showed that both factors (age and protease concentration) were significantly affected the hatchability based on p-value less than 0.005.

TABLE I: THE ANOVA STUDIES OF HATCHABILITY USING CRUDE PROTEASE

| Source of variation | F | p-value | Fcrit |
|---|---|---|---|
| Age factor | 145.2863 | 1.26E-10 | 4.351243 |
| Protease concentration | 75.01577 | 9.42E-12 | 2.866081 |
| Interaction | 33.8958 | 1.2E-08 | 2.866081 |

### D. $LC_{50}$ and $LC_{95}$ Studies

The lethal concentration, $LC_{50}$ for 3 and 9 days old eggs were 3.29U/ml and 3.54U/ml, respectively, while $LC_{95}$ for 3 and 9 days old eggs were 19.11 U/ml and 20.09U/ml (Table II). It showed a linear correlation between protease concentration and the egg's age. The concentration of protease should be high in order to suppress the hatchability since the embryo had already developed completely.

TABLE II: LETHAL CONCENTRATION ($LC_{50}$ AND $LC_{95}$) FOR 3 AND 9 DAYS OLD EGGS

| Age | $LC_{50}$ | $LC_{95}$ |
|---|---|---|
| 3 days old | 3.29 | 19.11 |
| 9 days old | 3.54 | 20.09 |

### E. Effect of Commercial Protease on the Hatchability of 3 (Three) and 9 (Nine) Days Old of P. Canaliculata Eggs

The effect of commercial protease treatment on the hatchability of 3 (three) and 9 (nine) days old of *P. canaliculata* eggs was investigated as shown in Fig. 3.

It showed that the hatchability was inversely proportionally correlated to the protease concentration. Apparently, the hatchability trend is similar to that of crude protease hatchability studies as shown in Fig. 3. At low protease concentrations (2.5 – 5 U/ml), the hatchability for 9 days old eggs were higher compared to 3 days old eggs. In this condition, the development of the embryo for 9 days old eggs had almost completed. Therefore, they hatched regardless of cuticle alteration compared to the 3 days old eggs which the cuticle were still immatured. However, at high concentration of crude protease (7.5 – 10 U/ml) the hatchability became saturated for both 3 and 9 days old eggs. It seemed that, although the embryo development were almost complete, there was a possibility that the enzyme had successfully penetrated through the eggshell and digested the proteinaceous yolk so as to disrupt further embryo development.

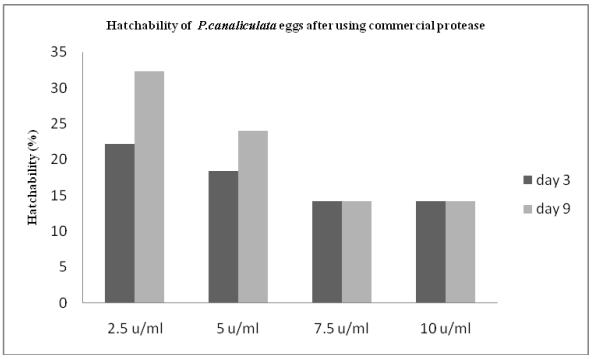


Fig. 3. Effect of commercial protease treatment on the hatchability of 3 (three) and 9 (nine) days old of *P.canaliculata* eggs.

## IV. CONCLUSION

The present studies showed that *A. oryzae* which was propagated through SSF using locally available and cheap raw material had excreted extracellular protease of higher activity compared to that excreted by *B. subtilis* and *B. thuringiensis* which were both propagated through SMF. It was also showed that the crude protease from *A. oryzae* at the concentration of 20.8U/ml had successfully suppressed the hatchability of *P. canaliculata* eggs as much as 30% and 20% for 3 and 9 days old eggs respectively. It can be concluded that protease produced from *A. oryzae* through SSF using locally available raw materials is a good candidate for biopesticide application to suppress the *P. canaliculata* eggs.

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