Optimization of dissolved proteins in extracts of earthworm (*Lumbricus rubellus*) with factor adding concentration of papain enzyme and earthworms

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Abstract. Earthworms (*Lumbricus rubellus*) can be used as an alternative to meet protein needs. This study aimed to obtain optimal N-Amino and Total Soluble Solid (TSS) results in earthworm extract. Efforts to improve the results of N-Amino and TDS (Total Dissolved Solids) in earthworm extract can be done by hydrolyzing the juice of earthworms with the help of enzyme protease papain under certain conditions, so it will facilitate the process of solving protein content. The experimental design in this study was Surface Methodology Response (RSM) model using thirteen models and two factors, namely the addition of papain enzyme concentration (6%, 8% and 10% (b/v)) and the percentage of earthworm base material (10%, 20% and 30% (b/v)). The results of this study showed the highest total protein value of 47.93% (g/L) obtained at the concentration of earthworms by 30% and 10% of papain enzyme. Then the validation results showed the optimum solution at 30% concentration of earthworms and 10% of papain enzyme that produced an N-Amino response of 7.2% and a TDS of 74% (g/L) with a Desirability of 0.906. N-Amino has a quadratic model with the actual variable equation, and TDS has a quadratic model with the actual variable equation.

1. Introduction

Earthworms (*Lumbricus rubellus*) is one of the most widely cultivated worms in Indonesia. The earthworm cultivation is relatively easy, requires little space, and has a relatively short production cycle; therefore, maintenance management is uncomplicated [1]. *Lumbricus rubellus* has various benefits and high potential to be developed due to its high crude protein content of 48.5% -61.9% [2]. The protein content of earthworms is higher than the healthy beef (21%) [3], yeast protein by-product (55%) [4] and soybeans (40%) [5]. According to Ministry of Agriculture Republic Indonesia (2019), earthworms can be used as an alternative to meet the demand for protein [6]. Earthworms are generally utilized as medicine, bio-feed supplements, cosmetic ingredients, organic fertilizers, and culture media. However, the utilization of them for microorganism growth culture media has not been widely found in Indonesia.

Protein from earthworms also has the potential as a nutrition source for microbial growth. Hidayat et al. (2018) found that culture media from earthworm juice (*L. Rubellus*) has good potential for *Saccharomyces cerevisiae* because of its nitrogen content which functions as a nutrient for microbial growth.
The dissolved protein content in culture media derived from earthworm extracts could be increased by hydrolysis using a protease enzyme. Ramakrishnan et al. (2013) found that the protein extraction process can be maximized using the protease enzyme to break down the undissolved protein components [8]. According to Baehaki et al. (2015), enzyme hydrolysis is more beneficial than chemical hydrolysis [9]. It is because enzymatic hydrolysis does not break the peptides and amino acids present in the protease enzyme. One of the protease enzymes used is the papain enzyme brand "PAYA," with a specific activity of 1.5093 units/gram. This enzyme has been commercially traded and is easy to obtain so that the price is relatively lower than other proteolytic enzymes.

The papain enzyme can dissect polypeptide chains by hydrolyzing the peptide bonds into simpler compounds [9]. The main factors that affect the hydrolysis process are temperature and pH [10]. According to Gupta et al. (2016), the factors that influence the enzymatic hydrolysis process are the enzyme characteristics, substrate type, chemical composition, and enzyme ratio to the protein substrate [11]. The concentration of the papain enzyme can affect the process of transforming protein into simpler elements. Therefore, the higher the concentration of the enzyme papain given, the higher the protein content produced. Anggraeni et al. (2017) discovered that the addition of 10% papain enzyme could produce a protein content of tuna by 48.6% [12].

Another factor that affects protein dissolution is the concentration of the raw material used. The number of raw materials can affect the number of components in the solution. The higher the concentration of earthworm material, the more protein accumulates and breaks down [7]. Sutama et al. (2015) used the earthworm solution with different concentrations of 10%, 20%, 30%, and 40%, which produced the most significant dissolved protein at a percentage of 30%, namely 4.39% [13]. Based on this description, this study aimed to obtain the optimal concentration of the enzyme papain and earthworms (as raw material) which can produce optimal N-Amino and Total Dissolved Solids (TDS) from earthworm extracts.

2. Material and methods

2.1. Materials
Earthworms (*Lumbricus rubellus*) were freshly collected from CV. Organic RAJ in Sukun District, Malang City, Indonesia. Other ingredients are distilled water (Hydrobath), papain enzyme powder (PAYA), 1M NaCl (Technical), 1M NaOH (Technical), NaOH (PA), 70% alcohol (technical), Phosphate Buffer solution pH 7 (technical), PP indicator 1% (technical), formaldehyde solution (technical), potassium oxalate (Mercck), filter cloth, filter paper no. 41 (Whatman), label paper and tissue.

2.2. Preparation of 6% salt solution
The salt solution preparation was referred to Sadiyah *et al.* (2018), which produces a solution with the optimal salt concentration for earthworm extraction (*Lumbricus rubellus*) [14]. Then the salt is weighed as much as 120 grams and was mixed with 2000 ml of distilled water to produce a 6% salt solution.

2.3. Preparation of earthworm (*Lumbricus rubellus*) juice
Preparation of the earthworm juice was referred to [14]. Earthworms were washed thoroughly from the soil and dirt and then drained. One thousand grams of earthworm mashed in a blender for 2-3 minutes at medium speed. 200 ml of water was added to the earthworm crushing process to accelerate the process. Crushed earthworms were mixed with 800 ml of 6% salt solution and stirred until homogeneous. The earthworm juice formed was considered a 100% concentration and was further diluted into several concentrations. Dilution of earthworm juice was carried out by drawing 12 mL (5.86%); 20 mL (10%); 40 mL (20%); 60 mL (30%); 68 mL (34.14%) and for control variables 20 mL (10%); 40 mL (20%); 60 mL (30%). Then for each dilution, a 6% salt solution was added until the volume reached 200 ml in each treatment.
2.4. Process of dissolving papain enzyme
This process was referred to [15]. The material used was crude papain enzyme marketed commercially under the brand name 'PAYA.' The crude papain enzyme was weighed following the treatment, namely 2.58 grams (5.172%); 3 grams (6%) 2 times; 4 grams (8%) 7 times; 5 grams (10%) 2 times, and 5.41 grams (10.828%). The enzyme was then put into a beaker glass and added with a solution of Buffer Phosphate pH 7 (40 ml) and distilled water (10 ml) with a ratio of 5:1 to each treatment. It was then stirred until homogeneous and left for 30 minutes before being mixed into earthworm juice.

2.5. Dissolving earthworm protein and papain enzyme
This process was referred to the combination of modified methods from [9] and [16]. A total of 160 mL of earthworm juice mixed with papain enzyme solution with a concentration of 0% (as a control); 5.172%; 6%; 8%; 10%; 10.828% (w/v) as much as 40 mL and stirred until homogeneous. The pH of the solution was adjusted to reach 7 by adding 1M NaOH or 1M HCL (±1mL). The solution was then stirred with a hot magnetic stirrer for 10 minutes at 60°C and 150 rpm. Samples were incubated for 24 hours at room temperature (20-25°C) in closed conditions. The sample was then heated at 105°C for 10 minutes and centrifugated at 8000 rpm at 4°C for 20 minutes. The resulting filtrate was analyzed for the value of total protein, N-Amino, and TDS (Total Dissolved Solids).

2.6. Analysis
Analysis of research data using Design Expert 10.0.1 software. Data were entered in a 2-factor Centralized Composite Design with 2 responses, namely Dissolved Protein (N-Amino) and TDS (Total Dissolved Solids). The results of the optimal solution from the results of data processing using Design Expert 10.0.1 can be validated by passing the earthworm (Lumbricus rubellus) dissolution process according to the factor treatment of the predicted optimal solution produced. The validation result of the optimal solution is by comparing the predicted response value obtained in the study and the actual value obtained from determining the factors in the computing system. The experimental design contains independent variables and coding in the centralized composite design is carried out as shown in Table 1.

Table 1. Independent variables and coding in centralized composite design.

| No | Variable’s code | Variable | Response |
|----|----------------|----------|----------|
|    | X<sub>1</sub> | X<sub>2</sub> | A= earthworms (%) (b/v) | B=Papain enzyme (%) (b/v) | Y<sub>1</sub>= N-Amino (%) (g/L) | Y<sub>2</sub>= TPT (g/L) |
| 1  | -1 | -1 | 10 | 6 | Y<sub>1</sub><sub>1</sub> | Y<sub>2</sub><sub>1</sub> |
| 2  | 1 | -1 | 30 | 6 | Y<sub>1</sub><sub>2</sub> | Y<sub>2</sub><sub>2</sub> |
| 3  | -1 | 1 | 10 | 10 | Y<sub>1</sub><sub>3</sub> | Y<sub>2</sub><sub>3</sub> |
| 4  | 1 | 1 | 30 | 10 | Y<sub>1</sub><sub>4</sub> | Y<sub>2</sub><sub>4</sub> |
| 5  | -1,414 | 0 | 5,857 | 8 | Y<sub>1</sub><sub>5</sub> | Y<sub>2</sub><sub>5</sub> |
| 6  | 1,414 | 0 | 34,142 | 8 | Y<sub>1</sub><sub>6</sub> | Y<sub>2</sub><sub>6</sub> |
| 7  | 0 | -1,414 | 20 | 5,172 | Y<sub>1</sub><sub>7</sub> | Y<sub>2</sub><sub>7</sub> |
| 8  | 0 | 1,414 | 20 | 10,828 | Y<sub>1</sub><sub>8</sub> | Y<sub>2</sub><sub>8</sub> |
| 9  | 0 | 0 | 20 | 8 | Y<sub>1</sub><sub>9</sub> | Y<sub>2</sub><sub>9</sub> |
| 10 | 0 | 0 | 20 | 8 | Y<sub>1</sub><sub>10</sub> | Y<sub>2</sub><sub>10</sub> |
| 11 | 0 | 0 | 20 | 8 | Y<sub>1</sub><sub>11</sub> | Y<sub>2</sub><sub>11</sub> |
| 12 | 0 | 0 | 20 | 8 | Y<sub>1</sub><sub>12</sub> | Y<sub>2</sub><sub>12</sub> |
| 13 | 0 | 0 | 20 | 8 | Y<sub>1</sub><sub>13</sub> | Y<sub>2</sub><sub>13</sub> |
3. Results and discussion

3.1. N-Amino response to earthworm extracts

The levels of N-Amino from the extract of earthworms (*Lumbricus rubellus*) added with the enzyme papain ranged from 1.37% to -7.67% (g/L). Treatment of 30% earthworm juice and 10% papain enzyme produced the highest N-Amino of 7.67% with the titration method and a total protein of 47.93%. Treatment of 5.857% earthworm juice and 8% papain enzyme produced the lowest N-Amino of 1.37% and a total protein of 8.54%. The results of the analysis of variance ANOVA on the N-Amino response obtained the following equation:

\[ Y_1 = 10.66 - 0.025X_1 - 2.38X_2 + 0.007X_1X_2 + 0.004X_1^2 + 0.15X_2^2 \]  

\( Y_1 = \text{N-Amino} \text{ (% (g/L), } X_1 = \text{Earthworm (b/v), } X_2 = \text{Papain enzyme (b/v)} \)  

The results of the polynomial equation show that each coefficient on the addition of earthworm concentration (\(X_1\)) has a higher value than the papain enzyme (\(X_2\)). The results show that the N-Amino yield was likely influenced by the addition of the concentration of earthworms (\(X_1\)). It may be due to more hydrolyzed protein sources. Rosida et al. (2014) found that an increase in the proportion of soybean substrate would increase the protein content of the tauco [17]. In addition, the chances of protein hydrolysis reaction will increase due to the addition of the papain enzyme so that the N-Amino content will also increase [18]. A three-dimensional response surface graph showing the relationship between the concentration factor of earthworms and the papain enzyme with their interaction on the N-amino response is presented in Figure 1.

![Figure 1. N-amino response three-dimensional surface graph.](image)

The slope of the graph in Figure 1 shows that the addition of the earthworms concentration can increase the N-Amino yield. In contrast, the variations in the addition of the enzyme papain do not show a significant difference and the graph results show a relatively flat pattern. This graph shows that the range of the slope between the earthworm concentration factor is higher than the papain enzyme concentration factor. The concentration of earthworms more influences the increase in N-Amino. The addition of the earthworms concentration displayed an increasing result on the N-Amino response. According to Hasibuan et al. (2015), variations in the earthworms concentration will affect the N-Amino levels [13]. The increase in the levels of the raw material used will increase the protein content being hydrolyzed. The hydrolysis process will remodel the protein bonds to become charged (NH\(^{3+}\) and COO) and the molecular protein weight will be reduced; thus, the protein solubility is increased [19].

The relationship between the addition of papain enzyme concentration and the N-amino response in Figure 1 shows a flat pattern. It might be caused by the concentration of the papain enzyme that has reached a saturation point to hydrolyze the earthworms substrate. According to Nurhayati et al. [19].
one of the main factors affecting enzyme activity is substrate concentration [20]. Fachraniah et al. (2002) also supported, they found that the competition between the substrate with peptide bonds could make the substrate fail to be hydrolysed [4]. The result is a decrease in the resulting N-Amino. According to Iskandar and Desi (2009), enzymatic hydrolysis can be influenced by the enzymatic reaction rate [21]. In general, the rate of the enzymatic reaction is influenced by substrate concentration.

The higher the substrate concentration, the faster the enzymatic reaction and then reaches a constant point. It shows that the enzymatic reaction rate has reached the maximum limit. Thereby the hydrolysis process does not work optimally. In addition, other factors that can influence are the characteristics of the enzyme, the type of substrate, chemical composition, and the ratio of the enzyme to the protein substrate used [11]. Iskandar and Desi (2009) found that 8% enzyme bromelain with 10 hours incubation time on Sardinella Lemuru fish substrate produced the highest dissolved protein [21]. It is because the enzyme reaction rate will increase as the enzyme concentration increases. The reaction rate in protease enzymes can occur to a certain extent and will tend to be constant with increasing enzyme concentrations. It occurs because the protease enzyme does not work optimally. As a result, the enzyme's ability to catalyze takes longer. Substrate concentration can also affect it. Based on the explanation above, it can be concluded that the addition of excessive enzyme concentrations will not have a significant effect on the N-Amino response because there is no additional substrate during the hydrolysis process, and the available substrate has been hydrolyzed [22].

3.2 Total dissolved solids (TDS) response on earthworm extracts

The TDS results from earthworm extracts treated with the addition of papain enzyme, and the concentration of earthworms had a value between 58 – 74 g/L. The highest TDS value of 74 g/L was obtained from 20% earthworm juice and 10.828% papain enzyme. Meanwhile, the lowest TDS was 58 g/L obtained from 10% earthworm juice and 6% papain enzyme. The equation obtained from the ANOVA analysis of the TDS response is as follows:

\[ Y_2 = 33.33 + 1.323X_1 + 2.66X_2 - 0.012X_1X_2 - 0.024X_1^2 - 0.043X_2^2 \] (3)

\[ Y_2 = \text{Total dissolved solids (g/L)}, \quad X_1 = \text{Earthworm (%)} (b/v), \quad X_2 = \text{Papain enzyme (%)} (b/v) \] (4)

The polynomial equation shows that each coefficient on the addition of papain enzyme concentration (X2) has a higher value than earthworms (X1). It indicates that the TDS is more influenced by the addition of the enzyme papain concentration (X2). These results follow Rosida et al. (2014), who showed that TDS would increase with the increasing concentration of the protease enzyme [17]. The high ability of the protease enzyme can break down several components so that it is easy to hydrolyze. The study results in the form of a three-dimensional response surface graph are presented in Figure 2.

The graph model in Figure 2 shows that increasing the concentration of earthworms and the enzyme papain can increase the TDS response. It is suspected that this occurs because the higher concentrations of earthworms and papain enzymes will result in higher TDS. When viewed from the slope range, the papain enzyme concentration factor is higher than the earthworm concentration factor, so that the papain enzyme concentration affects the TDS response more. It follows the research of Nafi et al. (2014), which stated that the addition of the papain protease enzyme could increase the TDS of the mushroom hydrolyzate [23]. Protease enzymes could break peptide bonds into simpler molecules to increase the number of polar groups produced. The increase in polar groups will make bonds with water easier to form so that the concentration of the dissolved material will increase and reduce precipitation. The increase in TDS could also be caused by the high ability of the enzyme to break down the substrate complex components into simpler components [17]. Based on this description, the resulting TDS response is more influenced by the papain enzyme concentration factor. It is following the polynomial equation. It can occur because hydrolysis can work optimally with the help of the papain enzyme, thereby increasing the TDS value.
The addition of the earthworms concentration also affected the increase in the TDS value. TDS in earthworm extract contains several components such as protein, carbohydrates, calcium, phosphorus, and iron [24]. According to Ismawati et al. (2016), the higher the addition of the substrate, the higher the TDS value [25]. It is suspected that another factor that could increase TDS is the metabolic activity of microorganisms that grow and develop in earthworm juice. According to Sadiyah et al. (2018), microorganisms in earthworm substrates can use protein as a source of nutrients [14]. Hence, the increase in TDS is thought to be derived from the metabolism of these microorganisms. Therefore, the hydrolysis process still produces precipitates in total sugar, protein, cellulose, and organic acids, which come from the metabolic activities of microorganisms and can be calculated as the TDS value [26].

![Figure 2. TDS response three dimensional surface graph.](image)

3.3 Optimization of N-Amino response and total dissolved solids
Optimization was carried out after the mathematical model of each response was obtained. Similar research and literature are needed to set standards on the optimization process's factor constraints and desired responses. Standard limits were determined using Design Expert 10.0.1 software. The objective of the factor was "is in range" at an earthworm concentration of 10% - 30% (w/v), papain enzyme concentration, the results of N-Amino and TDS. The optimal solution is presented in Table 1.

| Parameter                  | Prediction of standard |
|----------------------------|------------------------|
| Earthworm (%) (b/v)        | 30                     |
| Papain enzyme (%) (b/v)    | 10                     |
| N-Amino (%) (g/L)          | 7.56                   |
| TDS (g/L)                  | 69.71                  |
| Desirability               | 0.906                  |
| Information                | Selected               |

Table 1. Optimal result solution.

Table 1 shows the prediction of the optimal solution from Design Expert 10.0.1. The optimal solution produced was the addition of 30% earthworms and 10% papain enzymes. The combination of these factors produced a predictive value of the N-Amino parameter of 7.56% and a TDS of 69.71g/L with a desirability value of 0.906.
3.4 Validation of the optimum conditions for model prediction results
Validation of the optimum conditions is required to test the accuracy and ensure the optimal conditions suggested by the computational results. In this study, validation was carried out by comparing the validation results in the laboratory with the results of the solutions or predictions provided by the Design-Expert software. After that, the deviation value was determined. The results of the predicted and validated values in the laboratory are presented in Table 2.

| Parameter       | Lower prediction | Prediction | Highest prediction | Validation | Different | Deviation (%) |
|-----------------|------------------|------------|--------------------|------------|-----------|---------------|
| N-Amino (%)(g/L)| 6,26             | 7,56       | 8,86               | 7,2        | 0,36      | 5             |
| TDS (g/L)       | 63,54            | 69,71      | 75,89              | 74         | 4,29      | 5,79          |

Table 2 shows that the validation results of the N-Amino response were 7.2%, and the TDS response was 74 g/L. This validation value is considered eligible in the computing system because it is still in the range of predicted values.

4. Conclusions
Optimization of N-Amino and TDS in dissolving earthworm extract resulted in optimal predictive values of 7.56% and 69.71 g/L with a desirability value of 0.906. These conditions were obtained from the use of 10% papain enzyme and 30% earthworms. The validation results of the optimal solution produced a value of 7.2% for the N-Amino response and 74 g/L for the TPT response. The deviation for each parameter based on the validation results has a value of 5% for the N-Amino response and 5.79% for the TPT response where the validation results are considered eligible in the computing system because they are still in the range of predicted values. In the N-Amino response, the actual variable polynomial equation $Y_1 = 10.66 - 0.025X_1 - 2.38X_2 + 0.007X_1X_2 + 0.004X_1^2 + 0.15X_2^2$, while the response TPT results obtained equation $Y_2 = 33.33 + 1.323X_1 + 2.66X_2 - 0.012X_1X_2 - 0.024X_1^2 - 0.043X_2^2$.

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