Optimization of Hydrolytic Enzyme Production from Tempeh Starter to Increase In Vitro Pepsin Digestibility of Black Soldier Fly Maggot

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Abstract. This research aims to evaluate the optimal level and incubation time for the production of hydrolytic enzymes from tempeh starter (TS), in order to increase the in vitro pepsin digestibility of Black Soldier Fly (BSF), *Hermetia illucens,* maggot flour. The materials used were commercial tempeh starter, pepsin, and 15 days of age dried BSF maggot. In this study, two experiments were conducted. In a factorial completely randomized design, the analysis of variance (ANOVA) was performed to assess the significance of treatment effects, and orthogonal polynomial contrasts were employed to identify specific linear and quadratic trends among the group means. In experiment 1, research was carried out to determine the optimal starter level and incubation time to produce hydrolytic enzymes from tempeh starter. In experiment 2, crude enzyme from the best treatment in the first stage was used to increase the pepsin digestibility of BSF maggot flour. The optimal protein content and enzyme activities of tempeh starter was 0.10-0.13% starter with an incubation time of 2.00-3.47 days. Meanwhile, the optimal pepsin digestibility of BSF maggot flour was 1.51% enzymes with a hydrolysis time of 23.87 hours. This analysis measurement of protein content and enzymatic activity of tempeh starter and in vitro digestibility of BSF flour represent the preliminary methodology used to initially select hydrolyzed BSF flour to be a source of protein in animal feed.

Keywords: activity, enzymes, hydrolytic, digestibility, tempeh

Abstrak. Penelitian ini bertujuan untuk mengevaluasi taraf dan waktu inkubasi optimum untuk produksi enzim hidrolitik dari starter tempe (TS), dalam rangka meningkatkan kecernaan dalam pepsin in vitro tepung maggot black soldier fly (BSF) Hermetia illucens. Bahan yang digunakan adalah starter tempe komersial, pepsin dan maggot BSF kering umur 15 hari. Dalam penelitian ini, dilakukan dua kali percobaan. Metode penelitian untuk masing-masing percobaan adalah rancangan acak lengkap faktorial dengan uji lanjut ortogonal polinomial. Pada percobaan 1, penelitian dilakukan untuk menentukan taraf starter dan waktu inkubasi optimum untuk menghasilkan enzim hidrolitik dari starter tempe. Pada percobaan 2, ekstrak enzim kasar dari perlakuan terbaik pada tahap pertama digunakan untuk meningkatkan daya cerna pepsin maggot BSF. Kadar protein dan aktivitas enzim starter tempe optimum pada taraf starter 0,100,13% dengan waktu inkubasi 2,00-3,47 hari. Kecernaan dalam pepsin mencapai optimum pada taraf enzim 1,51% dengan waktu hidrolisis 23,87 jam. Analisis pengukuran kadar protein dan aktivitas enzimatik starter tempe serta daya cerna tepung BSF secara in vitro ini merupakan langkah awal untuk menyeleksi tepung BSF terhidrolisis sebagai sumber protein hewani dalam pakan. ternak.

Kata kunci: aktivitas, enzim, hidrolisis, kecernaan, tempe

Introduction

Hydrolytic enzymes can catalyze hydrolysis events and are involved in the reduction of proteins, lipids, and carbohydrates to their most basic structural components (Matias et al., 2021). Hydrolytic enzymes are widely utilized for industrial purposes such as food processing, pharmaceutical production, paper making, textiles, and oil refining (Thapa et al., 2019). Hydrolytic enzymes for the digestion of feed or livestock products have been extensively studied in the livestock industry, i.e. protease for meat tenderization, lipase to improve the aroma of cheese (Khan & Selamoglu, 2020), amylase to increase carbohydrate digestibility (Khasa et al., 2022), and fibrolase to reduce feed crude fiber and improve poultry production performance (Azrinnahar et al., 2021).

Some plants, microbes, and animals are known as hydrolytic enzymes manufacturer (bacteria, fungi, yeast). Microorganisms are the primary producers of hydrolytic enzymes due to

their rapid growth rate, ease of cultivation, high activity and stability in enzyme production, and ease of modification or genetic engineering (Kacobas et al., 2022). Yeast and fungi contribute more than 50% to the production of hydrolytic enzymes for industrial purposes. Meanwhile, bacteria contribute 30%, animals 8%, and plants around 4% (Thapa et al., 2019). The potential of fungi to produce hydrolytic enzymes, useful in the livestock sector for improving the pepsin digestibility of Black Soldier Fly (BSF) maggots, is evident in tempeh starter.

Tempeh starter (Rhizopus sp.) is a fungus from the Zygomycota phylum, long recognized and utilized in Indonesia as a starter for making tempeh. Tempeh starter is relatively cheap and easy to obtain on the market. It has been proven to produce hydrolytic enzymes, namely protease and lipase (Nugraha et al., 2022), as well as amylase (Freitas et al., 2014), on various growth substrates. Meanwhile, the BSF maggot is an insect that has the potential to become an alternative protein source in poultry feed, substituting fish meal or soybean meal, which need to be imported at relatively expensive prices (Hidayat, 2018). BSF has the benefits of an easy production system, fast growth, and efficient conversion of organic waste into highly nutritious biomass. BSF maggots contain 30.12% crude protein, with 53.76% essential amino acids and 46.24% non-essential amino acids (Miron et al., 2023), as well as monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), multivitamins, and minerals (El-Hack et al., 2020). Despite their high nutritional value, BSF maggots have shortages of high crude fiber 20.27% (Nafisah et al., 2019), high crude fat 25.78% (Zozo et al., 2022), and their exoskeleton contains 9.5% chitin polysaccharide (Soetemans et al., 2020). These conditions can disrupt feed consumption, digestibility of feed protein, and become an obstacle to the use of BSF maggot flour as poultry feed. One way to overcome the shortage of BSF maggots is hydrolysis with enzymes from tempeh starter. Therefore, the objective of this study was to determine the optimal starter level and incubation time for producing hydrolytic enzymes from tempeh starter, aiming to enhance the in vitro pepsin digestibility of BSF maggot flour.

Materials and Methods

Materials

Commercial tempeh starter Raprima (PT Aneka Fermentasi Indonesia) and 15 days old BSF maggot (PT Green Prosa) were purchased from the marketplace. The following sources provided the reagents utilized in this study: Brilliant blue G-250 (Sigma 27815), ethanol 95% (Merck K2557171), phosphoric acid 85% (Mallinckrodt), casein Hammarsten bovine (Merck 2242.0100), tris hydroxymethyl aminomethane (Merck 1.08382.0100), hydrochloric acid (Merck 1.00317.2500), trichloroacetic acid (Merck 1.00807.0250), sodium carbonate (Merck 1.06392.0500), Folin-Ciocalteu's (Merck 1.09001.0500), bovine serum albumin (Sigma A3608), 3,5-dinitrosalicylic acid (Sigma D0550), potassium sodium tartrate tetrahydrate (Sigma S2377), sodium metabisulfite (Sigma S9000), acetone (Merck 1.00014.2500), phenolphthalein (Merck 1.07233.0025), sodium hydroxide (Merck 1.06498.1000), dipotassium hydrogen phosphate (Merck 1.05104.1000), potassium dihydrogen phosphate (Merck 1.04873.1000), porcine pepsin (Merck 1.07185.0100).

Design of Experiment 1

Experiment 1 aimed to determine the optimal starter level and incubation time of tempeh starter for maximizing protein content and hydrolytic enzyme activity. The experiment used a 3 x 5 factorial completely randomized design with 3 replications, followed by further testing with orthogonal polynomial analysis. The first factor was the starter level: A1 = 0% tempeh starter, A2 = 0.1% tempeh starter, A3 = 0.2% tempeh starter. The second factor was the

incubation time: B1 = 1 day, B2 = 2 days, B3 = 3 days, B4 = 4 days, B5 = 5 days.

Cell-Free Filtrate Production. 15 days old BSF maggot were dried in an oven at 60 °C for 24 hours then blended into flour. The enzyme production medium was prepared by dissolving 2 g of BSF maggot flour in 150 ml of distilled water, followed by sterilization at 121 °C for 30 minutes. Tempeh starter (0%, 0.1%, and 0.2% w/v) was added to the production medium aseptically. The production medium was incubated in a shaking water bath for 5 x 24 hours at a temperature of 40 °C and an agitation of 50 rpm. Everyday liquid sampling was carried out and centrifuged at 5000 rpm for 10 minutes to obtain a cell-free filtrate which was used to measure protein content and the activity of protease, amylase, lipase, cellulase, and chitinase enzymes.

Protein Content. Cell-free filtrate protein content was measured using Bradford reagent at a wavelength of 595 nm (Bollag and Edelstein, 1996). The protein content of the samples was obtained using a regression from a 2% bovine serum albumin (BSA) standard curve.

Protease Activity. Protease activity was measured using a 2% Hammerstein casein substrate at a wavelength of 578 nm (Walter, 1984). Protease activity was obtained using the regression from the tyrosine standard curve. The results are then multiplied by the dilution factor and divided by the incubation time.

Amylase Activity. Amylase activity was measured using a 0.5% starch substrate and a 3,5-dinitrosalicylic acid (DNS) solvent at a wavelength of 540 nm (Bernfeld, 1955). Amylase activity was obtained using the regression from the 2% maltose standard curve.

Lipase Activity. Lipase activity (LA) was measured using a 2% (w/v) olive oil substrate by titration using 0.05 M NaOH and a phenolphthalein indicator (Kojima et al., 1994). The live oil substrate was made by mixing 50 ml of olive oil with 150 ml of polyvinyl alcohol (2% w/v) with vigorous shaking using a hand mixer. Lipase activity is obtained using the following formula. U/mg/minute used as unit

$$\mathrm{LA} = \frac{(\text{ml sample tirant} - \text{ml blank titrant}) \text{ x M NaOH x } 1000}{\text{ml sample volume x incubation time}}$$

Cellulase Activity. Cellulase activity was tested using a Whatman filter paper substrate number 1 and a DNS solvent at a wavelength of 550 nm (Camassola and Dillon, 2012). Cellulase activity was obtained using a regression from a 1% glucose standard curve.

Chitinase Activity. Chitinase activity (ChA) was tested using a 0.3% colloidal chitin substrate using the chitin reduction method (Arnold and Solomon, 1986). The remaining chitin in the reaction mixture was read using a spectrophotometer at a wavelength of 660 nm. U/mg/minute used as unit

$$\mathrm{ChA} = \frac{\frac{\text{sample absorbance} - \text{blank absorbance}}{0.001}}{60}$$

Design of Experiment 2

Experiment 2 was designed using data from Experiment 1 to assess the optimal enzyme level and hydrolysis time for improving the in vitro pepsin digestibility of BSF flour. The experiment used a 4 x 3 factorial completely randomized design with 3 replications, followed by further testing with orthogonal polynomial analysis. The first factor was the enzyme level: A1 = 0% enzyme, A2 = 1% enzyme, A3 = 2% enzyme, A4 = 3% enzyme. The second factor was the hydrolysis time: B1 = 0 hour, B2 = 24 hours, B3 = 48 hours.

Enzyme Production and Precipitation. The tempeh starter inoculum was prepared for producing hydrolytic enzymes. A total of 100 mL of distilled water was added to 2 g of BSF flour (2% w/v), then sterilized at 121 °C for 30 minutes. The 1.5 g of tempeh starter (0.12% w/v of 1000 ml final production medium), was added aseptically. The inoculum was incubated in a shaking water bath at 40 °C and 80 rpm for 12

hours. The enzyme production medium was made by mixing 900 ml distilled water with 18 g of BSF flour (2% w/v), then sterilized at 121 °C for 30 minutes. The tempeh starter inoculum from the previous preparation as much as 100 ml was added aseptically. The enzyme production medium was incubated in a shaking water bath at 40 °C and 80 rpm for 4 days. The liquid medium was centrifuged at 5000 rpm for 10 minutes to obtain a cell-free filtrate. The cell-free filtrate was then precipitated with 50% ammonium sulfate, left overnight at refrigerator temperature, and centrifuged at 10,000 rpm for 30 minutes. The pellet was taken and resuspended in phosphate buffer at a 1:1 ratio.

Hydrolysis of BSF Maggot. A total of 100 g of maggot flour were added to 100 ml of 0.05 M phosphate buffer, pH 7 (1:1) for each treatment unit. Precipitated enzymes (0%, 1%, 2% and 3%) (w/v) were added to each treatment aseptically, then incubated in an incubator at a temperature of 40 °C for 0, 24, and 48 hours. Samples of hydrolyzed maggots at each incubation time were taken and dried.

In Vitro Pepsin Digestibility. In vitro pepsin digestibility (IvPD) was tested using pepsin, HCl, and NaOH for pH conditioning (Prastika et al., 2019). The pepsin digestibility of the sample was measured by testing the protein content with Bradford method.

$$\%\ IvPD = \frac{Protein\ sample}{Protein\ total} x\ 100$$

Results and Discussion

Experiment 1

Table 1 presents the interaction between starter level and incubation time on protein content and the activities of protease, amylase, lipase, chitinase, and cellulase.

Protein Content

The protein content of the TS enzyme crude extract varied from 0.045 ± 0.001 to 0.062 ± 0.010 mg/ml. The analysis of variance revealed that while the interaction between starter level and incubation time did not significantly affect the protein content of the TS enzyme crude extract (P>0.05), both starter level and incubation time individually had a significant impact (P<0.05) on the protein content. The single effect of starter level is presented in Figure 1 with the equation $y = 0.0244 + 0.4705x - 1.6061x^2$ and a coefficient of determination value of 1. The starter level reached its optimum at point P (0.15%; 0.06 mg/ml). The single effect of incubation time is presented in Figure 2 with the equation $y = 0.0317 + 0.0097x - 0.0015x^2$ and a coefficient of determination value of 0.84. The incubation time reached its optimum at point P (3.23 days; 0.047 mg/ml) or the equivalent of 77.52 hours. The research results are different from Pratami et al., (2022) who found that the hydrolysis of benguk tempeh substrate with TS produced an optimal protein content of 37.08 mg/ml at a starter level of 0.20% and an incubation time of 144 hours.

The individual effects of starter level and incubation time influence the protein content of the TS enzyme crude extract. Increasing the starter level enhances opportunities for microorganisms to grow and develop further

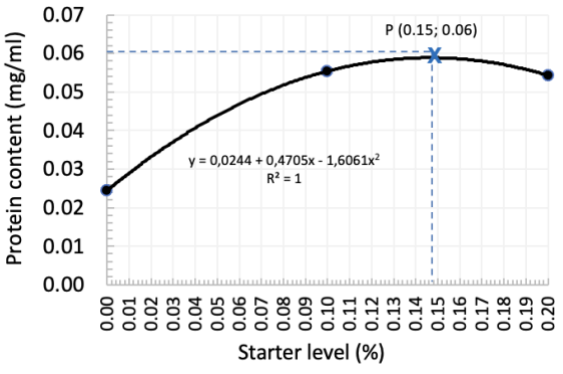

Figure 1. Effect of starter level on protein content of TS enzyme crude extract

Table 1. Starter level and incubation times on protein content and enzymes activity of tempeh starter

Parameters	Control					0.10%					0.20%					Sig.		
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	S	T	S x T
Protein content (mg/ml)	0.02	0.03	0.03	0.02	0.02	0.04	0.06	0.06	0.06	0.05	0.05	0.05	0.06	0.05	0.05	**	*	ns
Protease activity (U/mg/minute)	0.11	0.12	0.13	0.15	0.16	0.27	0.32	0.32	0.40	0.32	0.25	0.28	0.25	0.27	0.21	**	ns	ns
Amylase activity (U/mg/minute)	0.12	015	0.14	0.14	0.12	0.16	0.25	0.16	0.14	0.07	0.21	0.26	0.14	0.12	0.05	ns	**	ns
Lipase activity (U/mg/minute)	4.55	4.31	4.64	6.46	6.52	19.5	24.7	16.2	21.4	9.04	19.8	24.0	14.1	17.1	5.73	**	**	**
Chitinase activity (U/mg/minute)	1.53	1.46	1.51	1.63	1.71	1.59	2.24	1.89	4.01	4.09	0.55	1.31	1.68	3.06	2.21	**	**	**
Cellulase activity (U/mg/minute)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ns	ns	ns

Notes: S = starter level (0,0.1,0.2%), T = incubation time (1-5 days), ns = non-significant

The individual effects of starter level and incubation time influence the protein content of the TS enzyme crude extract. Increasing the starter level enhances opportunities for microorganisms to grow and develop further. According to Montesqrit et al. (2020), the higher the level of starter used, the faster the fermentation process and the higher the protein content to a certain extent. The increase in protein contents is caused by the secretion of extracellular proteins and enzymes by microorganisms for cell metabolic processes (Anigboro et al., 2022). Increasing the incubation time to a certain extent causes the cell division of microorganisms to increase. During the incubation time, carbohydrates in the substrate will be hydrolyzed as a carbon source for the growth of microorganisms and produce complex proteins, peptides, and free amino acids resulting in an increase in protein contents (Garcia et al., 2022). As incubation time increases, the nutritional content of the substrate becomes more limited, which slows down microorganism growth and results in a decrease in protein content.

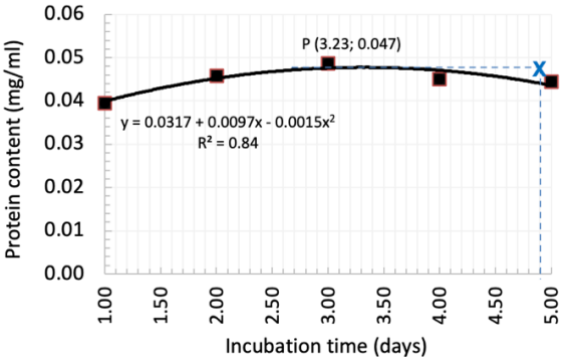

Figure 2. Effect of incubation time on protein content of TS enzyme crude extract

Protease Activity

TS protease activity ranged from 0.207 ± 0.032 to 0.398 ± 0.101 U/mg/minute. Analysis of variance indicated that neither the interaction between starter level and incubation time nor the effect of incubation time alone had a significant impact (P>0.05) on TS protease activity. However, the starter level alone had a

significant effect (P<0.05) on TS protease activity. The effect of starter level is presented in Figure 3 with the equation y = 0.1335 + 3.215x - 13.185x^2 with a coefficient value determination 1. The starter level reaches its optimum at point P (0.12%; 0.33 U/mg/minute). The single influence of hydrolysis time is presented in Figure 4 with the equation y = 0.1655 + 0.0487x - 0.007x^2 with a coefficient of determination value of 0.56. The incubation time reached its optimum at point P (3.48 days; 0.25 U/mg/minute) or the equivalent of 83.52 hours.

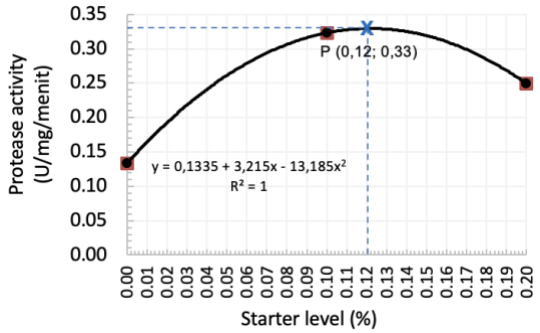

Figure 3. Effect of starter level on protease activity of TS

In this study, TS protease activity aligns with Rahayu et al. (2019), where soybean tempeh fermented with Rhizopus oligosporus at a 0.2% level was first detected at 24 hours of incubation, marked by the growth of white mycelia. The activity peaked at 96 hours (0.046 U/ml) and then declined due to the appearance of black spores, indicating fungal decay and reduced growth. Handayani et al. (2020) showed that hydrolysis of jack beans using TS *Rhizopus oligosporus* at a 0.3% level for 48 hours produced an optimal protease activity of 0.02 U/ml.

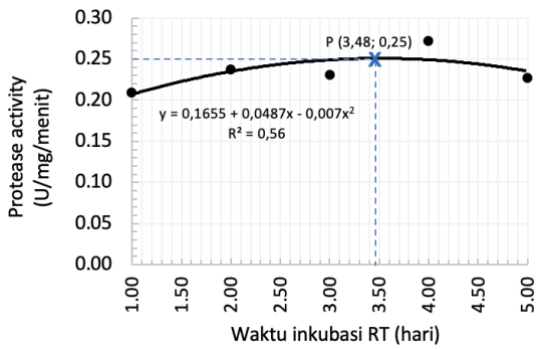

Figure 4. Effect of incubation time on protease activity of TS

The starter level influences the enzyme activity of microorganisms. Montesqrit et al. (2022) found that a higher starter level accelerates the hydrolysis process, leading to an increase in enzyme production. Incubation time also affects the enzyme activity of microorganisms. According to Alrumman et al. (2018), a longer incubation time allows microorganisms to carry out more cell divisions, resulting in more optimal enzyme production. The protease activity detected in the control treatment could be caused by the activity of hydrolytic enzymes found in the digestive tract of BSF maggots which are used as enzyme production media. According to Kim et al. (2021), BSF maggot small intestine extract showed trypsin, leucine arylamidase, α-galactosidase, β-galactosidase, α-mannosidase, and α-fucosidase activities. Besides that, research by Bonelli et al. (2020) showed that the intestinal contents of BSF maggots detected trypsin and chymotrypsin activity. This protease enzyme is produced by the BSF maggot in response to the protein content contained in the substrate in which it lives.

Amylase Activity

TS amylase activity ranged from 0.048 ± 0.006 to 0.259 ± 0.098 U/mg/minute. The analysis of variance revealed that neither the interaction between starter level and incubation time nor the effect of starter level alone significantly affected TS protease activity (P>0.05). However, incubation time alone has a significant effect (P<0.05) on TS protease activity. The single effect of starter level is presented in Figure 5 with the equation y = 0.1365 + 0.2833x - 0.9167x^2 with a coefficient of determination value of 1. The starter level reaches its optimum at point P (0.15%; 0.158 U/mg/minute). The single effect of hydrolysis time is presented in Figure 6 with the equation y = 0.1419 + 0.0474x - 0.0122x^2 with a coefficient of determination value of 0.82. Incubation time reached its optimum at point P (1.94 days; 0.19 U/mg/minute) or the equivalent

of 46.56 hours. The research results contrast with those of Freitas et al. (2014), who reported that the tempeh starter Rhizopus oligosporus on a wheat flour substrate achieved optimal amylase activity of 3.71 U/ml at a 0.2% starter level and an incubation time of 96 hours. The hydrolysis of tapioca pulp substrate with tempeh starter *Rhizopus oryzae* at a starter level of 0.7% and an incubation time of 168 hours produced an optimal specific amylase activity of 4.69 U/mg (Martgrita et al., 2023).

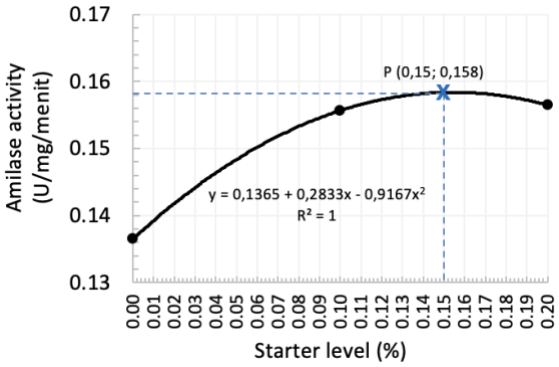

Figure 5. Effect of starter level on amilase activity of TS

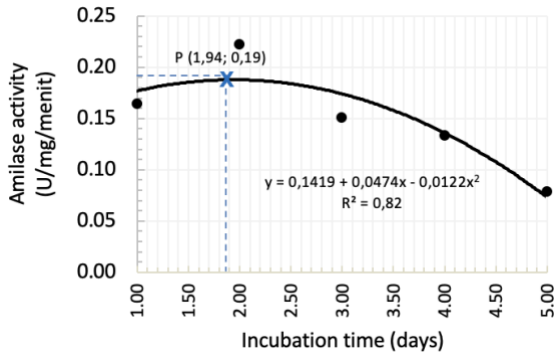

Figure 6. Effect of incubation time on amylase activity of TS

The amylase enzyme activity observed in the control treatment may be attributed to the digestive enzyme activity of the BSF maggot substrate. Sun et al. (2021) found significant lipase, esterase, cellulase and amylase enzyme activities in the small intestines of BSF maggots. Bonelli et al. (2020) stated that the detection of the amylase enzyme in the midgut juice of BSF maggot was a response to the carbohydrate content in the feed. According to Kresnawati et al. (2019), amylase is an extracellular enzyme that is released into the environment by bacterial cells to break down starch into monosaccharides and disaccharides which are then used by cells for growth. Microorganisms will produce extracellular enzymes if an inducer is present in the substrate on which they grow. Black soldier fly maggot substrate contains 21.47% carbohydrate (Andari et al., 2021). The carbohydrate content in the substrate acts as an inducer for tempeh starter microorganisms to produce the amylase enzyme.

Lipase Activity

TS lipase activity ranged from 5.735 ± 0.896 to 24.692 ± 0.570 U/mg/minute. Analysis of variance showed that the interaction of starter level and incubation time had a significant effect ($P<0.01$) on TS lipase activity. Further orthogonal polynomial tests indicated that the interaction between starter level and incubation time had a quadratic effect on increasing TS lipase activity, as shown in Figure 7. The starter level reaches its optimum at point P (0.068%; 16.00 U/mg/minute), while the incubation time achieves its peak at point Q (2.35 days; 16.00 U/mg/minute), which is equivalent to 56.40 hours. The results of the study showed higher activity than *Rhizopus oryzae* TS lipase activity on cooking oil substrates with a starter level of 8% and an incubation time of 96 hours which was 1.40 U/mg (Helal et al., 2021).

The interaction of starter level and incubation time affects TS lipase activity. The results indicated that lipase activity increased up to the optimal point (where points P and Q intersect), after which it decreased as both the starter level and incubation time were further increased. This can be caused by the accumulation of end products which will then inhibit enzyme production. According to Gendi et al. (2021), the accumulation of the final product of an enzyme will end the enzyme's activity in a process called feedback inhibition. This process serves as a cell regulatory mechanism to control the rate of

metabolic reactions, preventing the excessive accumulation of end products and adjusting the production of substances based on the cell's needs. According to Sada et al. (2021), the higher the level of starter used on a substrate, the denser the fungal growth will be, resulting in a decrease in enzyme activity due to the rapidly decreasing nutrients in the substrate. Incubation time influences the growth of fungal mycelia, where Rhizopus oryzae experiences a logarithmic growth phase at an incubation time of 42 hours, a stationary phase up to 48 hours, and a death phase above 48 hours. Research by Alrumman et al. (2019) showed that the growth of Bacillus licheniformis entered a lag phase for 24 hours, a logarithmic phase for up to 48 hours, a stationary phase for up to 72 hours, and continued with a death phase for up to 96 hours.

The lipase activity detected in the control treatment was caused by the presence of hydrolytic enzymes in the digestive tract of BSF maggots. Bonelli et al. (2020) demonstrated that lipase enzyme activity was present in the lumen contents of BSF maggots fed a diet with a crude fat content of 2.7%, but was not detected in maggots fed a vegetable mixture with a fat content of 0.7%. This shows that the lipase activity in the control treatment is a response of the hydrolytic enzymes of the maggot digestive tract to the fat substrate in their feed

Chitinase Activity

TS chitinase activity ranged from 0.547 ± 0.306 to 4.092 ± 0.349 U/mg/minute. Analysis of variance shows that the interaction of starter level and incubation time has a significant effect (P<0.01) on TS chitinase activity. Further orthogonal polynomial tests show that the interaction of starter level and incubation time has a quadratic effect on increasing TS chitinase activity as shown in Figure 8. The starter level achieves its optimum at point P (0.154%; 2.50 U/mg/minute), while the incubation time reaches its peak at point Q (4.10 days; 2.50 U/mg/minute), which is equivalent to 98.40 hours. The research results are different from Baihaqi et al. (2022), who found that the hydrolysis of soybeans using *Rhizopus oligosporus* produced an optimal specific chitinase activity of 4.01 U/mg with a starter level of 1% and an incubation time of 72 hours.

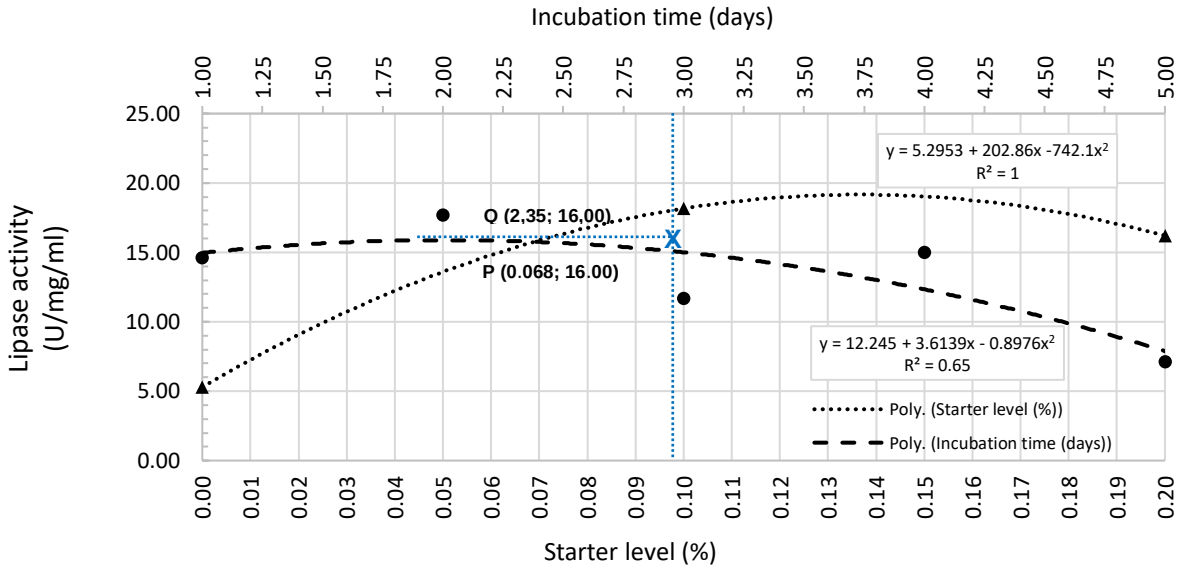

Figure 7. Interaction of starter level and incubation time on TS lipase activity

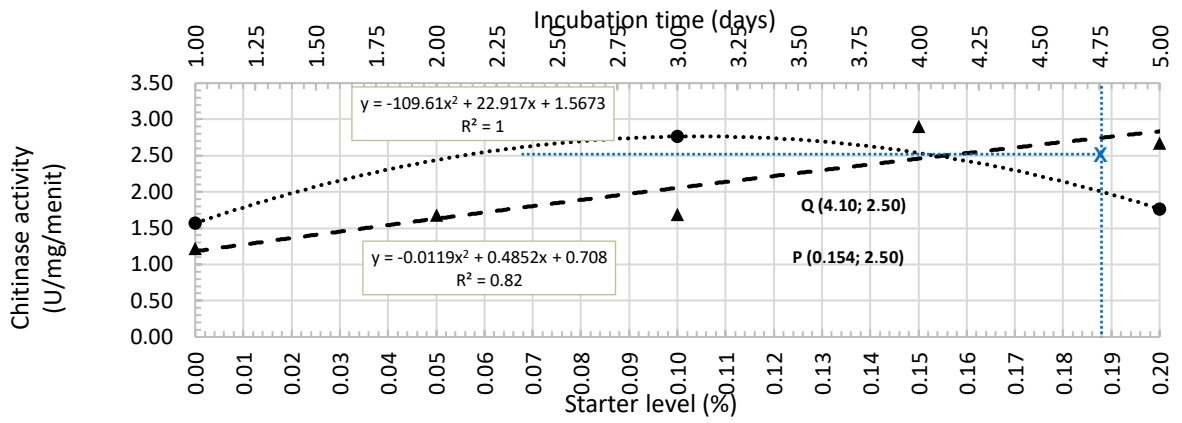

Figure 8. Interaction of starter level and incubation time on TS chitinase activity

The interaction between starter level and incubation time affects the activity of TS chitinase microorganisms. The results showed that chitinase activity increased to the optimal point (where points P and Q met), then chitinase activity decreased as the starter level increased. However, TS chitinase activity continued to increase with increasing incubation time. This indicates that within 5-days of incubation, TS has not yet fully optimized the utilization of the chitin present in the substrate. Chitin is a complex carbohydrate that can be hydrolyzed by chitinolytic microorganisms as an energy source for cells (Aslamsyah et al., 2018). However, if the substrate contains simple carbohydrates, the microorganisms will utilize it first.

Cellulase Activity

Tempeh starter cellulase activity was not detected in this study. The cellulase enzyme is an extracellular protein. Extracellular proteins are adaptive, so their synthesis will only occur if an inducer is present (Rohmah et al., 2019). The inducer in question is cellulose contained in the production media. The absence of cellulase enzyme activity from tempeh starter indicates that the BSF larvae production media does not contain cellulose. According to Thomas et al. (2018), the synthesis of cellulase by microorganisms requires a cellulose substrate as an inducer, which prompts the production of cellulase enzyme to hydrolyze cellulose. However, if the substrate contains simple carbohydrates such as glucose and fructose, microorganisms will use these for their metabolism instead, and they will not produce cellulase enzyme as it is unnecessary.

Experiment 2

The enzymes used in experiment 2 were obtained from the best treatment in the first experiment as seen from the activity of protease, lipase, and chitinase enzymes. Based on the activity of these three enzymes, the best treatment for TS enzyme production was obtained with a starter level of 0.12% and an incubation time of 4 days. This combination yields TS enzyme activities of 0.034 U/mg/minute for protease, 5.73 U/mg/minute for lipase, and 0.11 U/mg/minute for chitinase.

BSF In Vitro Pepsin Digestibility

The pepsin digestibility of TS maggot hydrolysate ranged from 75.09 ± 0.93% to 85.06 ± 0.54%, as shown in Table 2. Analysis of variance shows that the interaction of enzyme level and hydrolysis time has a significant effect ($P<0.05$) on the pepsin digestibility of TS maggot hydrolysate. Further orthogonal polynomial tests show that the interaction of enzyme level and hydrolysis time has a quadratic effect on increasing the pepsin digestibility of TS maggot hydrolysate as presented in Figure 9. The enzyme level reaches its optimum at point P (0.12%; 77.20%), while the hydrolysis time achieves its peak at point Q (2.00 hours; 77.20%).

Table 2. Enzyme level and hydrolysis times on in vitro pepsin digestibility of BSF maggot

Parameters	Control			Enzyme 1%			2%			3%			Sig.		
	0	24	48	0	24	48	0	24	48	0	24	48	E	H	S x T
Pepsin digestibility (%)	75.25	83.65	70.57	76.80	85.06	79.45	76.33	82.88	77.42	74.62	81.78	75.09	*	*	**

Notes: E = enzyme level (0,1,2,3%), H = hydrolysis time (0-48 hours)

There is an interaction between enzyme levels and hydrolysis time affecting the solubility of BSF maggot pepsin in vitro. As the enzyme level increases and the hydrolysis time extends, the digestibility decreases. Like inhibitory power and antioxidant activity, pepsin solubility can also decrease due to protein damage from metabolic conditions that exceed the optimal limit. Zaefarian et al. (2021) stated that in vitro tests using gastric and intestinal digestion models vary greatly and are not standardized. Digestibility depends on timing and enzyme activity, so longer digestion times ideally result in greater nutrient digestibility. The transit time of feed in the chicken's digestive tract is 30-90 minutes from the proventriculus to the ventriculus and 75-110 minutes in the small intestine.

Maggot hydrolysate exhibits higher pepsin solubility compared to non-hydrolyzed maggot due to its simpler protein molecular structure. According to Pasaribu (2018), the increase in digestibility of fermented feed protein is caused by the degradation of protein into amino acids by microbes, thereby enhancing digestibility. The research results were higher than other feed ingredients that play the same role as an alternative protein source for poultry, namely the algae Spirulina sp. with a pepsin solubility of 52.35% (Pootthachaya et al., 2023) and hydrolyzed chicken feathers of 30.20% (Rahayu et al., 2014). The solubility value of pepsin for various feed ingredients varies depending on the quality of the substrate and the specificity of the enzyme. Chicken feather protein is mostly composed of keratin, which is difficult to hydrolyze by proteases in general, so it has a lower solubility in pepsin than BSF maggot hydrolysate.

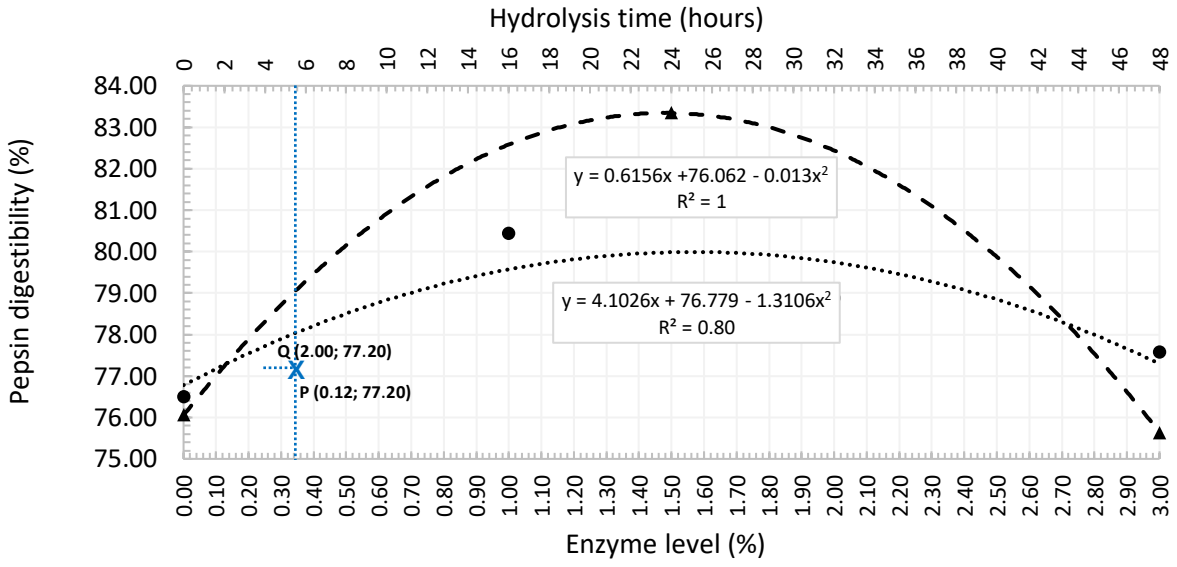

Figure 9. Interaction of enzyme level and hydrolysis time on TS maggot hydrolysate pepsin digestibility

Conclusions

The interaction between starter level and incubation time can enhance both the protein content and enzyme activity of tempeh starter (TS). Protein content and TS enzyme activity increased at the starter level from 0.10% to 0.13% with an incubation time of 2.00 to 3.47 days. The interaction between enzyme levels and hydrolysis time can enhance the quality of BSF maggot flour. The quality of BSF maggots hydrolyzed by the TS enzyme improved at an enzyme level of 1.20% with a hydrolysis time ranging from 23.87 to 2.00 hours.

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