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Fibrinolytic Protease Production: Impact of Initial pH and Temperature in Solid-State Fermentation by *Rhizopus microsporus* var. *oligosporus* FNCC 6010

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Abstract

Background*: Fibrinolytic enzyme is one of the cardiovascular disease therapies. Rhizopus microsporus var. oligosporus is microorganism that has been evaluated to produce fibrinolytic protease by fermentation. This study conducted fermentation of helianthi annui semen (sunflower seed) by Rhizopus microsporus var. oligosporus to produce fibrinolytic enzyme.* ***Objective:*** *This study aims to determine the effect of Initial pH and incubation temperature and its optimization in the production of fibrinolytic protease by Rhizopus microsporus var. oligosporus FNCC 6010 in solid-state fermentation on helianthi annui semen (sunflower seed) substrate. Optimum condition was determined by maximum protease and fibrinolytic activity.* ***Method:*** *A crude enzyme of protease fibrinolytic was obtained from the supernatant extract of fermented sunflower seed. Protease activity was measured by the skimmed milk agar (SMA) plate method, and fibrinolytic activity was determined by the fibrin agar plate method.* ***Result:*** *It was found that the starting pH affects both the proteolytic and fibrinolytic activity of enzymes that are produced in fermentation. The starting pH of 5.0 showed higher fibrinolytic and proteolytic activity values compared to the starting pH of 7.0. The incubation temperature 33±1 °C had the higher activity compared to 28±1 °C or 37±1 °C.* ***Conclusion:*** *Initial pH and incubation temperature affect the proteolytic and fibrinolytic activity of crude enzyme extracted from fermented sunflower seed by Rhizopus microsporus var. oligosporus. The optimum condition for producing fibrinolytic protease in the state fermentation method was an initial pH of 5.0 and an incubation temperature of 33±1°C.*

Keywords*: fibrinolytic enzyme, protease, Rhizopus microsporus var. oligosporus, solid state fermentation*

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INTRODUCTION

Cardiovascular diseases (CVDs) such as stroke, high blood pressure, arrhythmia, peripheral vascular disease, and valvular heart disease are the leading causes of death (Altaf *et al.,* 2021). Thrombosis, or accumulation of fibrin clots in blood vessels, is one of the risk factors for cardiovascular disease. Cardiovascular disease is usually followed by the rupture of atherosclerotic plaques and erosion, which activates coagulated proteins, leading to thrombus formation (Kotb, 2014).

The fibrinolytic enzyme is one of the thrombosis treatments that belong to the group of proteases. It catalyses the breakdown of fibrin clots by converting the inactive plasminogen to active plasmin or directly lysing the fibrin into fibrin degradation products (Krishnamurthy *et al.,* 2018). Microorganisms like bacteria and fungi are sources of fibrinolytic protease. These microorganisms have benefits over plants and animals in that they may be grown in huge quantities and in a short amount of time using fermentation techniques (Miglani *et al.,* 2017).

Rhizopus microsporus var. oligosporus is a fungus commonly used in the manufacture of tempeh (Nout & Kiers, 2005). Fermentation by *R. oligosporus* produces a wide variety of enzymes, including proteases (Lim *et al.,* 2022). A previous study reported that *R. microsporus var. oligosporus* made fibrinolytic enzymes by fermentation (Sarao *et al.,* 2010). Other studies have revealed that *Rhizopus species* can produce fibrinolytic proteases, including *R. oryzae* (Sada *et al*., 2021); *Rhizopus chinensis* 12 (Xiao-Lan *et al.,* 2005); *R. microsporus var. tuberosus* (Zhang *et al.,* 2015); and *Rhizopus oligosporus* (Poernomo *et al.,* 2017).

The method commonly used to produce fibrinolytic enzymes from fungi is solid-state fermentation (SSF). Technically, solid-state fermentation (SSF) is used to produce microbial metabolites (Sambo *et al.,* 2021). This method is preferred rather than submerged fermentation (SmF) because the yield of enzymes in such cultivation systems increases significantly by 1.5 times greater (Muhammed *et al.,* 2020; Sharma *et al.,* 2021).

The SSF process is carried out on a solid substrate with certain environmental conditions. The substrate functions as a growth medium, which is a source of nutrients, including nitrogen, carbon, and minerals as activators and vitamins (Srivastava *et al.,* 2019; Ahamed *et al.,* 2022).

Sunflower seeds are rich in valuable compounds that are important as a fermentation media, such as protein, carbon, and nitrogen with the percentage of 21%, 44.23%, and 0.41%, respectively (Grasso *et al.,* 2020; Hermansyah *et al.,* 2019; Karefyllakis *et al.,* 2019). In the previous study, sunflower oil cake was used as a growth medium of Candida guilliermondii by solid state fermentation to produce fibrinolytic enzymes (Rashad *et al.,* 2012). Furthermore, another study reported that sunflower meal had been reported as a substrate for producing protease enzyme from *R. oligosporus* (Rauf *et al.,* 2010).

Initial pH and incubation temperature are the critical factors that affect enzyme production in solid state fermentation method (Srivastava *et al*., 2019). Initial pH has an important influence on the production of proteolytic enzymes because each microorganism grows best at its specific pH that exhibits its maximum metabolic activity (Kotb, 2015). The optimal pH of the medium helps in maintaining the homeostasis of electrical charge in the membrane and proteins in the medium by regulating proton pumps and transporting nutrients across the membrane *(*Muhammed *et al.,* 2020). pH also affects the enzymatic processes and transport of various components that support cell growth and enzyme formation (Rauf *et al.,* 2010). Buffers are commonly employed to maintain pH during microbial growth and are added to the media along with the nutrients required for growth.(Madigan *et al.,* 2015).

During the fermentation process, the incubation temperature has an impact on the metabolic activity of microorganisms and the growth of fungi (Rauf *et al.,* 2010). Temperature also affects the stability of the enzymes produced (Kotb, 2013; Sada *et al.,* 2021). Furthermore, an increase of incubation temperature causes a rise of enzyme activity up to a certain point, and after that, there can be an effect on the growth of microorganisms and allow enzyme denaturation to occur (Vinuthna & Raju, 2022). It was reported that *R. oligosporus* shows faster growth of mycelium at a temperature of 25 – 37 °C (Nout & Kiers, 2005).

This research aims to develop fermented helianthi annui semen by *R. microsporus var. oligosporus* FNCC 6010 to produce fibrinolytic enzymes in solid-state fermentation.The impact of starting pH and incubation temperature on enzyme synthesis will be examined. The proteolytic activity of the crude enzyme was assayed using the skimmed milk agar (SMA) plate method, and the fibrinolytic activity of the crude enzyme was determined using the fibrin plate method. The optimum Initial pH and incubation temperature of enzyme production will be defined by the higher proteolytic and fibrinolytic activity that the crude extract had.

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MATERIALS AND METHODS

Materials

R. microsporus var. oligosporus obtained from Food and Nutrition Culture Collection (FNCC) 6010, Universitas Gadjah Mada, Helianthi annui semen from House of Organix, Kec. Cilincing, Kota Jakarta Utara, Daerah Khusus Ibukota Jakarta, Potatoes Dextrose Broth (PDB) Himedia®, skimmed milk powder Nutrifood Indonesia, Nattokinase Swanson®, fibrin bovine Sigma®, agarose AA®, methylene blue Sigma-Aldrich, distilled water.

Equipment

Autoclave electric HL – 340 Series, Vertical Type Steam Sterilizer, Memmert Incubator, Genesys-20 Spectrophotometer, Sartorius Type BP 221S Digital Scales, Laminar Air Flow Cabinet, magnetic stirrer, Samsung refrigerator, ultracentrifuge HERMLE Z36HK, Hettich Zentrifugen EBA 20 centrifuge, Vortex Thermolyne Maxi Mix, Fisher Versamix pH-meter, micro pipette, Eppendorf, Eppendorf tube rack, Philips juicer.

Methods

Enrichment of *Rhizopus microsporus var. oligosporus* FNCC 6010

Enrichment of *R. microsporus var. oligosporus* was done in Potato dextrose agar (PDA) slant media by taken 1 Ôse from *R. microsporus var. oligosporus* FNCC 6010 culture stock, then the Ôse was scrapped into PDA slant and incubated in 33 ± 2 °C for 72 hours.

Rhizopus microsporus var. oligosporus spore suspension preparation

Ten mL of phosphate buffer 0,1M pH 5.00 or pH 7.00 is added into the culture of *R. microsporus var. oligosporus* on its tube. The tube is vortexed for ± 10 minutes until the spore of *R. microsporus var. oligosporus* released from the media. The optical density of spore suspension was measured to get transmittance ± 25% (λ 580) by spectrophotometer.

Fermentation and optimization process

Helianthi annui semen was washed three times and bubbled in distilled water for 25 minutes. The hull of the seeds was removed and doused in distilled water 1:3 (w/v) for 12 hours. At that point, the seed was re-boiled for 15 minutes. After that, the seed that had been boiled was cleared out, and dried at room temperature for 2 hours, and the moisture content test was done. The fermentation process was done by weighing 30 g of sunflower seed and putting it in a sterile container of 140 cm^2. Added 500 µL of *R. microsporus var. oligosporus* spore suspension in pH 5.0 or pH 7.0, spread on the seed, and incubated at various incubation temperatures for 24 hours. Both parameters such as initial pH, incubation temperature in the state fermentation method, were optimized in one variable-at-a-time strategy.

The extraction process obtained protease fibrinolytic crude enzyme

Fermented sunflower seed was weighed at 20 g, and 50 mL phosphate buffer 0.1M pH 7.00 was added. It was blended for 2 minutes and centrifuged for 30 minutes at 6000 rpm. The supernatant was used as the crude enzyme.

Proteolytic activity assay

The proteolytic activity assay was done using the skimmed milk agar (SMA) method. Skimmed milk agar media was prepared by dissolving 3 g skim milk in 30 mL distilled water and 3 g agar in 100 mL distilled water. Pour skim milk solution into agar solution and heat it. Put 20 mL media into each tube, then sterilize at 121 °C for 15 minutes. Pour skim milk agar media into a sterile petri dish. Proteolytic activity assay was done by pipetting a 40µl sample or control to each hole of skim milk agar plate and incubating the plate at 33±2°C for 20 hours. Nattokinase (100g/10mL) was used as the positive control, and phosphate buffer 0.1M pH 7.00 was used as the negative control (Poernomo *et al*., 2017). The proteolytic index was calculated by the equation below.

$$\text{Proteolytic index} = \frac{\text{average clear zone diameter (mm)}}{\text{media hole diameter (mm)}}$$

Fibrinolytic activity assay

A fibrinolytic activity assay was done by the fibrin plate method. The fibrin plate media was prepared by dissolving 1.5 g agar in 60 mL heated distilled water, and 0.5 g fibrin was added to the agar solution. Put one droplet pipette of methylene blue into fibrin media. The fibrin media was pasteurized for 3 minutes at 80±2 °C. About 20 mL of fibrin media was poured into a sterile petri dish method modified (Poernomo *et al*., 2021). Fibrinolytic activity assay was done by pipetting a 40µl sample or control to each hole of fibrin media on a petri dish. Incubated at 37±2°C for 24 hours. Nattokinase (100 g/10 mL) was used as the positive control, and phosphate buffer 0.1M pH 7.00 was used as negative control. The fibrinolytic index was calculated using the equation below.

$$\text{Fibrinolytic index} = \frac{\text{average clear zone diameter (mm)}}{\text{media hole diameter (mm)}}$$

Statistical analysis

Microsoft Excel (Office 2013, Microsoft) was used to calculate the average and standard deviation of both

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the proteolytic index and fibrinolytic index. The proteolytic index and fibrinolytic index from the data were analyzed using SPSS statistical software (SPSS version 25.0, SPSSInc., Chicago, IL, USA). Each data was performed by one-way ANOVA (analysis of variance) at a 5% confidence level to know if there was a significant difference from the index value. If there was significance, Tukey test was performed at 5% probability.

RESULTS AND DISCUSSION

The result of *R. microsporus var. oligosporus* enrichment on PDA media for 72 hours at a temperature of 33 ± 1 ° C is shown in Figure 1. PDA media is used because it was better compared to saborouds dextrose agar (SDA) based on the number of colonies grown in agar media (Poernomo *et al.*, 2017). The culture enrichment result on the PDA slant showed the forming of white hyphae with a black surface. Culture storage of *R. microsporus var. oligosporus* was at 4 °C, and it was rejuvenated every four weeks on a new PDA during research use, referring to previous studies (Vinuthna & Raju, 2022).

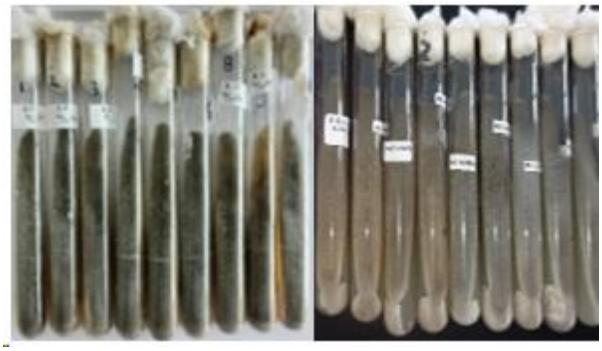

Figure 1. *Rhizopus microsporus var. oligosporus* FNCC 6010 culture on PDA slant with incubation time 72 hours and incubation temperature 33±2°C

R. microsporus var. oligosporus spore suspension was prepared by adding a phosphate buffer with a pH of 5.0 or pH 7.0 to the tube of culture until transmittance of T 25% at λ 580 nm was obtained. Percent transmittance was measured at λ580 because in the λ range, 400 – 600 nm is the appropriate wavelength for observing microbial cell turbidity, which is a method for measuring spore suspension (Madigan *et al.*, 2015). The cell suspension looks cloudy because the cells scatter light that passes through the suspension. Since cell mass is proportional to the number of cells, turbidity can be used to estimate cell count and the technique is widely used in microbiology (Madigan *et al.*, 2015). In this study, the transmittance value of each suspension of *R. microsporus var. oligosporus* was set to T 25% for each fermentation process to be reproducible. The number of cells used in the fermentation process was set to 10^7 CFU/mL (Poernomo *et al.*, 2017) because a lower number of cells can lead to longer incubation times and potential bacterial contamination. Furthermore, a higher number of cells may result in an excessive increase in temperature in the process and premature death of the fungus (Nout & Kiers, 2005).

Figure 2. Fermentation product of sunflower seed by *Rhizopus microsporus var. oligosporus* FNCC 6010 with (a) initial pH 5.0 and (b)pH 7.0; incubation time of 24 hours and incubation temperature 33 ± 2°C

Visually, Figure 2 and Figure 3 showed that the fermentation products after 24 hours were white and had a specific odour. The white colour of the fermentation product is caused by the growth of mycelia in the growth medium (Hariyono *et al.*, 2021). At variable pH of 5.0 and 7.0 and incubation temperature 33 ± 1°C and 24 h incubation time, the fermented product physically looked similar. In temperature variations, the differences could be observed at temperatures 28 ± 1 °C where the mycelia grew less frequently than at temperatures of 33 ± 1 °C and 37 ± 1 °C. Previous research explained that at a temperature of 25 °C or below, the rate of the fermentation process decreases so that it takes a longer time to achieve results equivalent to fermentation at a temperature of 30 °C or higher (Nout & Kiers, 2005). In addition, at low temperatures, there is a possibility that bacterial contamination grows faster than the growth rate of *Rhizopus sp.* (Dwiatmaka *et al.*, 2022).

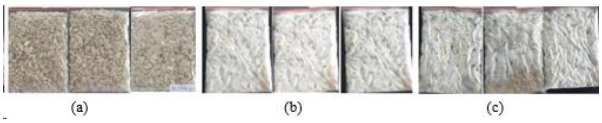

Figure 3. Fermentation product of sunflower seed by *Rhizopus microsporus var. oligosporus* FNCC 6010 with variations incubation temperature (a) 28±1°C; (b) 33±1°C; (c) 37±1°C; incubation time of 24 hours; initial pH fermentation of 5.0

The fermentation results were then extracted to obtain crude enzymes. In the solid-state fermentation method for enzyme production, fermentation results can

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be used directly as a source of crude enzyme and easily harvested from the media (Sambo *et al.,* 2021). In this study, the crude enzyme was defined as an enzyme obtained from the fermentation of sunflower seed by *Rhizopus microsporus var. oligosporus* FNCC 6010, which was extracted with a phosphate buffer solvent at pH 7.0 and took the supernatant. Extraction was carried out with a pH of 7.0 because previous studies have revealed that the activity and stability of fibrinolytic enzymes produced by *Rhizopus microsporus var. tuberosus* are optimal at pH 7.0 (Zhang *et al.,* 2015). An essential step in enzyme downstream processing is the removal of the cells, solids, and colloids from the fermentation media which commonly using the ultracentrifugation process (Raju & Divakar, 2014). Enzymes were stored at < 5°C to prevent microbial contamination and maintain enzyme activity and stability.

Figure 4. Proteolytic activity of crude enzyme from fermented sunflower seed with fermentation condition variation in initial pH 5.0 (S5 R1, S5 R2, S5 R3) and initial pH 7.0 (S7 R1, S7 R2, S7 R3).

It was found that crude enzymes from fermented sunflower seeds showed a clear zone around the hole media in skim milk agar (SMA) plates. Nattokinase as positive control also demonstrated a clear zone, while phosphate buffer 0.1M pH 7 as negative control did not appear in a clear zone, according to Figure 4. The clear zone showed the proteolytic activity by hydrolysis of the peptide bonds in casein that link amino acids together in the polypeptide chain in skimmed milk agar (Susanti *et al.,* 2019). The rising of protease activity is demonstrated by the higher clear zone in SMA media (Ahamed *et al.,* 2022)

In this study, the influence of the starting pH during the fermentation process was investigated at pH 5.0 and pH 7.0, incubation temperature of 33 1 °C, and an incubation period of 24 hours. The results showed that the value of the proteolytic index at pH 5.0 was higher than at pH 7.0, according to Table 1. Statistical analysis showed that there was a significant difference in proteolytic activity at initial pH 5.0 and pH 7.0 with Sig. 0.005 (< 0.05). Similar research showed that the protease production method in submerged fermentation by *Rhizopus microsporus var. oligosporus* is optimal at pH 5.5 (Sarao *et al*., 2010). Previous research has revealed that the optimum pH of the media for protease synthesis from *Rhizopus oligosporus* IHS13 in sunflower meal, wheat bran, and rice bran is at pH 5.0. At pH above and below 5.0, there is a decrease in enzyme production and so below that pH (Haq & Mukhtar, 2004). Another study used pH 5.5 to produce proteases from *Rhizopus oryzae* in bread media (Benabda *et al.,* 2019).

The results of fibrinolytic activity are described in Figure 5. showed that at the initial pH condition of fermentation, 5.0 obtained a fibrinolytic index greater than at pH 7.0, according to Table 2. That was a statistical difference from value Sig. 0.030 (< 0.05). It was indicated by a clear zone around the hole in fibrin agar media. Other studies have shown that the fibrinolytic activity of enzymes produced by *Serratia rubidaea* KUAS001 at the initial pH of enzyme production 5.0 is better than at pH 7.0, and decreases with increasing pH (Anusree *et al.,* 2020). Each microorganism grows best at its specific pH, where it exhibits maximum metabolic activity, the fungus showed optimal metabolic activity in the pH range of 4 to 5 (Srivastava *et al.,* 2019).

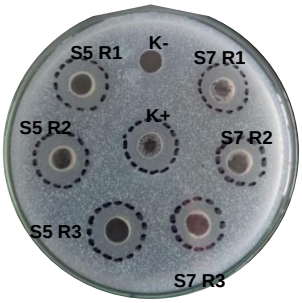

Figure 5. Fibrinolytic activity of crude enzyme from fermented sunflower seed with fermentation condition variation initial pH 5.0 (S5 R1, S5 R2, S5 R3) and initial pH 7.0 (S7 R1, S7 R2, S7 R3)

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Table 1. Proteolytic index of crude enzyme from fermented sunflower seed incubation time 24 hours at 33 ± 1°C initial pH variation

Sample/Control	Clear zone diameter (mm)	Mean clear zone diameter (mm) ± SD	Proteolytic index	Mean Proteolytic index ± SD
K+	14.03	14.03	2.00	2.00
K-	0.00	0.00	0.00	0.00
S5 R1	18.45	18.40 ± 0.19	2.64	2.63 ± 0.03
S5 R2	18.60		2.66	
S5 R3	18.15		2.59	
S7 R1	17.10	16.63 ± 0.41	2.44	2.38 ± 0.06
S7 R2	16.10		2.30	
S7 R3	16.10		2.39	

Note: K+ = positive control; K– = negative control; S5 R1, S5 R2, S5 R3 = crude enzyme from fermented sunflower seed in each pH 5 replication 1, 2 and 3; S7 R1, S7 R2, S7 R3 = crude enzyme from fermented sunflower seed in initial pH 7 replication 1, 2 and 3

Table 2. Fibrinolytic index of crude enzyme from fermented sunflower seed in initial pH variation

Sample/Control	Clear zone diameter (mm)	Mean clear zone diameter(mm)± SD	Fibrinolytic index	Mean Fibrinolytic index ± SD
K+	15.13	15.13	2.16	2.16
K-	0.00	0.00	0.00	0.00
S5 R1	15.13	15.29 ± 0.12	2.16	2.18 ± 0.02
S5 R2	15.30		2.19	
S5 R3	15.43		2.20	
S7 R1	14.70	13.82 ± 0.28	2.10	2.01 ± 0.07
S7 R2	13.43		1.92	
S7 R3	14.00		2.00	

Note: K+ = positive control; K– = negative control; S5 R1, S5 R2, S5 R3 = crude enzyme from fermented sunflower seed in initial pH of 5.0 replication 1, 2 and 3; S7 R1, S7 R2, S7 R3 = crude enzyme from fermented sunflower seed in initial pH 7.0 replication 1, 2 and 3

Further research was carried out on the variability of incubation temperature parameters of 28±1, 33±1, and 37±1 °C, initial pH 5.0, and incubation time of 24 hours. Based on the results of the proteolytic activity assay, shown in (Figure 6). it was found that the proteolytic index of the crude enzyme at an incubation temperature of 33±1 °C produced a larger value compared to temperatures of 28±1 °C and 37±1 °C according to Table 3. It was statistically different with the value Sig. 0.000 (<0.05). In enzyme production by microorganisms, an increase in the temperature of fermentation causes a rise in enzyme activity up to a certain point, and after that, there can be a decrease (Ahamed *et al*., 2022; Boratyński *et al*., 2018) in line with previous studies that revealed that there was an increase in protease activity produced by *Rhizopus oligosporus* IHS13 with substrates of wheat bran and rice bran at an incubation temperature of 25 °C to 30 °C then after that decreased at a temperature of 35 °C which showed that the organism was mesophyll (Haq & Mukhtar, 2004). Similar research on protease production by *Rhizopus oligosporus* shows that the optimal fermentation temperature is 30 °C, which then decreases to 35 °C (Rauf *et al.*, 2010).

Figure 6. Proteolytic activity assay result of crude enzyme fermented sunflower seed with fermentation condition incubation temperature 28 ±1 °C (S28 R1, S28 R2, S28 R3); 33 ±1 °C (S33 R1, S33 R2, S33 R3); 37 ±1 °C (S37 R1, S37 R2, S37 R3)

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Table 3. Proteolytic index of crude enzyme from fermented sunflower seed in various incubation temperature

Sample/Control	Clear zone diameter (mm)	Mean clear zone diameter (mm) ± SD	Proteolytic index	Mean Proteolytic index ± SD
K+	18.37	18.37	2.62 ± 0.02	2.62
K-	0.00	0.00	0.00 ± 0.00	0.00
S28 R1	14.00	14.53 ± 0.40	2.00	2.08 ± 0.06
S28 R2	14.63		2.09	
S28 R3	14.97		2.14	
S33 R1	16.93	17.01 ± 0.11	2.42	2.43 ± 0.02
S33 R2	16.93		2.42	
S33 R3	17.17		2.45	
S37 R1	15.77	15.74 ± 0.11	2.25	2.25 ± 0.02
S37 R2	15.60		2.23	
S37 R3	15.87		2.27	

Note: K+ = positive control; K– = negative control; S28 R1, S28 R2, S28 R3 = crude enzyme fermented sunflower seed with fermentation condition incubation temperature 28 ±1 °C; S33 R1, S33 R2, S33 R3= crude enzyme fermented sunflower seed with fermentation condition incubation temperature 33 ±1 °C; S37 R1, S37 R2, S37 R3= crude enzyme fermented sunflower seed with fermentation condition incubation temperature 37±1 °C

Table 4. Fibrinolytic index of the crude enzyme from fermented sunflower seed in various incubation temperature

Sample/Control	Clear zone diameter (mm)	Mean clear zone diameter (mm) ± SD	Fibrinolytic index	Mean Fibrinolytic index ± SD
K+	14.70	14.70	2.10	2.10 ± 0.05
K-	0.00	0.00	0.00	0.00 ± 0.00
S28 R1	13.23	12.78 ± 0.37	1.89	1.83 ± 0.05
S28 R2	12.77		1.82	
S28 R3	12.33		1.76	
S33 R1	15.90	15.67 ± 0.22	2.27	2.24 ± 0.03
S33 R2	15.73		2.25	
S33 R3	15.37		2.20	
S37 R1	14.13	14.33 ± 0.14	2.02	2.05 ± 0.02
S37 R2	14.40		2.06	
S37 R3	14.47		2.07	

Note: K+ = positive control; K– = negative control; S28 R1, S28 R2, S28 R3 = crude enzyme fermented sunflower seed with fermentation condition incubation temperature 28 ±1 °C; S33 R1, S33 R2, S33 R3= crude enzyme fermented sunflower seed with fermentation condition incubation temperature 33 ±1 °C; S37 R1, S37 R2, S37 R3= crude enzyme fermented *Helianthus annus L. semen* with fermentation condition incubation temperature 37±1 °C

Fibrinolytic activity assay results shown in (Figure 7) demonstrate that there was an increase in fibrinolytic activity in enzyme production with an incubation temperature from 28 ±1 °C up to 33 ±1 °C. Then, at the incubation temperature of 37 ±1 °C, the fibrinolytic activity of the crude enzyme was decreased, according to (Table 4) The results statistically showed a significant difference between temperature variations at the value Sig. 0.000 (< 0.05). Similar research results revealed that *Rhizopus oryzae* FNCC 6078 for fibrinolytic enzyme production increased with increasing incubation temperature, and the optimum temperature was reached at 35 °C (Sada *et al*., 2021). Environmental temperature is one of the factors that greatly affect the growth and metabolism of organisms (Srivastava *et al*., 2019). At low temperatures, rising temperatures increase the growth rate of organisms because the speed of reactions catalyzed by enzymes increases, so metabolism increases, resulting in ,more significant growth and production of enzymes by microorganisms. But the increase will reach a certain point until after that, too high temperatures can damage microorganisms by denaturation, transporters, other proteins, and plasma membranes. Temperatures too high for microorganisms promote stretching and eventual breakdown of weak hydrogen bonds in the enzyme, particularly in the

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protease enzyme generated during the fermentation process. (Haq & Mukhtar, 2004).

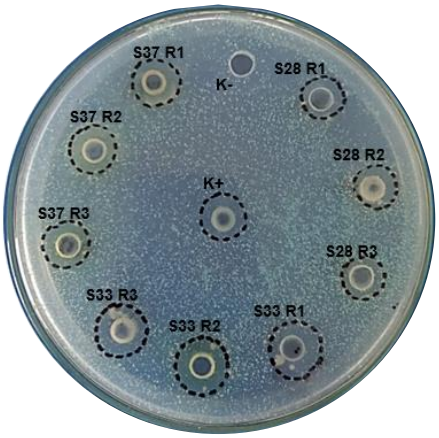

Figure 7. Fibrinolytic activity assay result of crude enzyme fermented sunflower seed with fermentation condition incubation temperature 28 ±1 °C (S28 R1, S28 R2, S28 R3); 33 ±1 °C (S33 R1, S33 R2, S33 R3); 37 ±1 °C (S37 R1, S37 R2, S37 R3)

CONCLUSION

The initial pH and incubation temperature for producing fibrinolytic protease from *Rhizopus microsporus var. oligosporus* FNCC 6010 on helianthi annui semen (sunflower seed) substrate in solid state fermentation affects both the proteolytic and fibrinolytic activity. The initial pH of 5.0 had higher activity than the initial pH of 7.0. The incubation temperature 33±1 °C had the higher activity compared to 28±1 °C or 37±1 °C so the optimum condition was an initial pH of 5.0 and incubation temperature of 33±1°C.

AUTHOR CONTRIBUTIONS

Conceptualization, R. L., A. T. P., A. S.; Methodology, R. L., A. T. P., A. S.; Software, R. L.; Validation, R. L.; Formal Analysis, R. L.; Investigation, R. L.; Resources, R. L., A. T. P., A. S.; Data Curation, R. L., A. T. P., A. S.; Writing - Original Draft, R. L., A. T. P., A. S.; Writing - Review & Editing, R. L., A. T. P., A. S.; Visualization, R. L., A. T. P., A. S.; Supervision, R. L., A. T. P., A. S.; Project Administration, R. L., A. T. P., A. S.; Funding Acquisition, R. L., A. T. P., A. S.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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