Borneo Journal of Pharmacy
Vol 6 Issue 3 August 2023
Pages 295 – 304

https://journal.umpr.ac.id/index.php/bjop/article/view/4665
DOI: https://doi.org/10.33084/bjop.v6i3.4665
e-ISSN: 2621-4814

Research Article

Fibrinolytic Protease Activity of Crude Enzyme from Fermented Sunflower (*Helianthus annuus*) and Common Bean (*Phaseolus vulgaris*) seeds by *Rhizopus microsporus* var. *oligosporus* FNCC 6010 in Solid State Fermentation

Rebhika Lusiana [1]

Achmad Toto Poernomo [2*]

Achmad Syahrani [2]

[1] Master Program of Pharmaceutical Sciences, Universitas Airlangga, Surabaya, East Java, Indonesia

[2] Department of Pharmaceutical Sciences, Universitas Airlangga, Surabaya, East Java, Indonesia

*email: achmad-t-p@ff.unair.ac.id

Keywords:
Fibrinolytic
Helianthus annuus L.
Phaseolus vulgaris L.
Protease
Rhizopus microsporus var. *oligosporus*

Abstract

In the entire world, cardiovascular diseases (CVDs) are the main cause of death. For the treatment of CVDs, microbial fibrinolytic enzymes are highly regarded as novel therapeutic candidates. This study was purposed to determine the fibrinolytic protease activity produced by fungus source, which is *Rhizopus microsporus* var. *oligosporus* FNCC 6010 in fermented sunflower (*Helianthus annuus*) seed and common bean (*Phaseolus vulgaris*) seed. Fermentation was carried out by solid-state fermentation method at an initial pH of 5, incubation temperature of 33±1°C, and incubation time of 24 hours. The fermented seed was extracted to obtain supernatant as the crude enzyme. The proteolytic activity assay was done by the skimmed milk agar (SMA) plate method to obtain the proteolytic index, and the fibrinolytic activity assay was conducted by the fibrin-agarose plate method to get the fibrinolytic index. The results show that crude enzymes from fermented *H. annuus* and *P. vulgaris* seeds by *R. microsporus* have fibrinolytic protease activity with proteolytic index 2.64 ± 0.01 and 2.23 ± 0.04, respectively. The fibrinolytic index is 2.40 ± 0.06 and 1.64 ± 0.06, respectively. Therefore, the crude enzyme has the potential to be further researched as a candidate for thrombolytic agents. The purification, characterization, and in-depth research are needed to develop enzymes into preparations for preventing and treating CVDs.

Received: January 28th, 2023
1st Revised: May 8th, 2023
2nd Revised: June 27th, 2023
Accepted: June 30th, 2023
Published: August 30th, 2023

INTRODUCTION

Thrombosis or fibrin aggregation in blood vessels leading to myocardial infarction or other cardiovascular diseases (CVDs) are the leading cause of death today[1]. One of the anti-thrombolytic therapies is fibrinolytic enzymes, for example, tissue plasminogen activator (t-PA), urokinase, streptokinase, and bacterial plasminogen activator[2]. Fibrinolytic enzymes belong to the group of proteases that can degrade fibrin[3]. Fibrinolytic enzymes are distinguished into two categories: the first is plasminogen activator (PA), which acts indirectly, and the second, which directly degrades fibrin into fibrin degradation product[4]. The first category is urokinase and streptokinase types, which convert plasminogen into plasmin and cause fibrin hydrolysis. The second category of examples is nattokinase, which directly hydrolyzes fibrin and breaks down the thrombus[5]. However, some fibrinolytic enzymes have several limitations, such as higher cost of production and side effects, including bleeding complications, gastric bleeding, and allergies[6,7]. It forces the researcher to find alternative and safer fibrinolytic enzymes for CVD treatment.

How to cite: Lusiana R, Poernomo AT, Syahrani A. Fibrinolytic Protease Activity of Crude Enzyme from Fermented Sunflower (Helianthus annuus) and Common Bean (Phaseolus vulgaris) seeds by Rhizopus microsporus var. oligosporus FNCC 6010 in Solid State Fermentation. Borneo J Pharm. 2023;6(3):295-304. doi:10.33084/bjop.v6i3.4665

Fibrinolytic enzymes can come from macroorganisms such as plants, snake venom, earthworms such as *Lumbricus rubellus*[1], sponges, and also come from microorganisms including bacteria such as *Bacillus subtilis* DC271 *Stenotrophomonas* sp.[8], algae such as *Ulva pertusa*, *Arthrospira platens*, parasites *Rhipicephalus microplus*[1], and fungus such as *Rhizopus* sp.[9-12]. There are some fermented foods by microbes that have been shown to produce fibrinolytic enzymes, such as *natto*, a traditional Japanese food derived from soybeans fermented by *B. subtilis*, *douchi* from China, *chung kook jang* from Korea, and *tempeh* from Indonesia[1]. Various sources of fibrinolytic enzymes from microbes have recently attracted attention due to their relatively low cost and can be produced on a large scale over plant and animal sources. In general, the enzymes produced by microbes are extracellular and secreted into the fermentation medium, making them easy to extract[13].

Rhizopus sp. has been reported to produce fibrinolytic proteases, including *R. oryzae*[9]; *R. chinensis*[10,12]; *R. microsporus* var. *tuberosus*[14]; and *R. oligosporus*. *Rhizopus microsporus* var. *oligosporus* is a fungus from *Rhizopus* sp. commonly used to manufacture *tempeh*[15]. *Rhizopus oligosporus* NRRL 2710 and NRRL 2549 show faster growth of mycelium. *Rhizopus oligosporus* can grow at 25-37°C in rice or cassava media[16]. Fermentation of *R. oligosporus* produces a wide variety of enzymes, including proteases[17]. *Rhizopus oligosporus* has been studied to produce protease enzymes in wheat bran, rice bran, and soybean media in solid-state fermentation (SSF) methods[3]. *Tempeh* fermentation of black soybeans by *R. oligosporus* produces fibrinolytic enzymes and has thrombolytic activity[12].

Fermentation is the process of breaking down large organic molecules with the help of microorganisms into simpler molecules[18] that are used to produce microbial metabolites[5]. The SSF process is carried out on a solid substrate with certain environmental conditions[19]. Substrate selection is essential in maximizing enzyme production from the fermentation process[2]. The substrate functions as a growth medium, a source of nutrients including nitrogen, carbon, minerals as activators, and vitamins[20]. Through the mycelium, the fungus penetrates the substrate to convert it into bioactive components[21].

Some substrates that can be used as a medium for metabolite production are sunflower (*Helianthus annuus*) and common bean (*Phaseolus vulgaris*) seeds. *Helianthus annuus* seed is a source of protein with a protein content 21%[22]. Carbon content is as much as 35.8%[23], nitrogen content is as much as 3.33-3.70%[24], and are rich in other valuable compounds[25,26]. Previous studies have shown that *H. annuus* seed extract produces several enzymes, including proteases[27]. *Helianthus annuus* meal has been reported as a growth medium for *R. oligosporus* to produce protease enzymes[28]. *Phaseolus vulgaris* seed is a source of protein, carbohydrates, vitamins, and minerals, unsaturated fatty acids[29]. The protein content varies between 16-33%. The organic content of carbon is 38.90% – 45.01%. The nitrogen content is 3.79-4.96%. Carbohydrates content in *P. vulgaris* seed is about 50-60%, and fat loss is 2.20-5.03%. Approximately nine peptides have been identified in *P. vulgaris* seed having protease activity[30,31].

To our knowledge, there has yet to be a study on the production of protease fibrinolytic from *R. microsporus* var. *oligosporus* with *H. annuus* and *P. vulgaris* seeds substrates in the SSF method. Both substrates contain substances that have protease activity[27,30] because fibrinolytic enzymes are inducible enzymes, so the incorporation of protease-rich substances induces the yield of enzymes produced[20]. Based on the problem, this study was purposed to determine the activity of a microbial protease fibrinolytic that was produced by *R. microsporus* var. *oligosporus* Food and Nutrition Culture Collection (FNCC) 6010 in *H. annuus* and *P. vulgaris* seeds substrate as novel therapeutic candidates as thrombolytic agent for CVDs treatment. The proteolytic activity of the crude enzyme produced was examined by the skimmed milk agar (SMA) plate method, and fibrinolytic activity was determined by the fibrin plate method.

MATERIALS AND METHODS

Materials

Rhizopus microsporus var. *oligosporus* FNCC 6010 was bought from Universitas Gadjah Mada, Indonesia. *Phaseolus vulgaris* seed (**Figure 1a**) was bought from CV. Aura Seed Indonesia, Bunut, Bringin, Badas, Kediri, East Java, Indonesia. *Helianthus annuus* seed (**Figure 1b**) was bought from House of Organix, Cilincing, North Jakarta, Jakarta Capital Special Region, Indonesia. Other materials including Potatoes Dextrose Broth (PDB) Himedia®, phosphate buffer 0,1 M (pH 5-7), skimmed milk powder Nutrifood Indonesia, Nattokinase Swanson®, fibrin bovine Sigma®, distilled water, agarose, and methylene blue. Instruments used include 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, micropipette, Eppendorf, Eppendorf tube rack, and Philips juicer.

a **b**

Figure 1. (**a**) *Phaseolus vulgaris* and (**b**) *Helianthus annuus* seeds.

Methods

PDA slant media and enrichment of R. microsporus

A potato dextrose agar (PDA) slant was used as media for the enrichment of *R. microsporus*. PDA slant was made by weighing 2.9 g of PDB powder and 1.5 g of agarose. One hundred mL of distilled water was added to the mixed powder, and then the solution was heated until soluble and homogenous. Twelve mL of solution was put into each tube and sterilized by autoclave at 121°C for 15 minutes, then left until compact. *Rhizopus microsporus* was enriched by inoculating the culture stock into a PDA slant and incubating it at 30±2°C for 72 hours.

Preparation of R. microsporus var. oligosporus suspension

A total of 10 mL of 0.1 M phosphate buffer pH 5.00 was added to *R. microsporus* var. *oligosporus* in PDA slant media, then vortex until the spores were separated from the medium and let stand for a few moments until the suspension was clear and the sediment was separated (±15 minutes). The optical density was then measured with a spectrophotometer to obtain a transmittance of ±25% (λ 580 nm).

Substrate preparation and fermentation process

Helianthus annuus and *P. vulgaris* seeds were used as substrates in controlling conditions. Each seed was thoroughly washed three times and boiled in distilled water for 25 minutes. The epidermis was removed, rinsed, and soaked in distilled water 1:3 (w/v) for 12 hours. Then, the mixture was re-boiled for 15 minutes. The mixture was left to dry at room temperature for two hours, and the water content was tested. The fermentation process was done by weighing 30 g of each prepared seed, adding 500 µL of *R. microsporus* var. *oligosporus* suspension, and spreading it on the seed. Incubated at 33±1°C for 24 hours.

Extraction process obtaining crude enzyme and crude extract

The crude extract was obtained from unfermented seeds, while crude enzyme was obtained from fermented seeds by weighing 20 g of each seed or fermented seed and adding 50 mL of 0.1 M phosphate buffer pH 7.00. The mixture was blended for two minutes and centrifuged for 30 minutes at 6000 rpm. The supernatant was used as a sample.

Skimmed milk agar media preparation and proteolytic activity assay

Skimmed milk agar media were prepared by dissolving 3 g skim milk in 30 mL distilled water and 3 g agar in 100 mL distilled water and pouring skimmed milk solution into agar solution and heated. A total of 20 mL media was put into each tube and then sterilized at 121°C for 15 minutes. The SMA media was put into a sterilized petri dish. The proteolytic activity assay was done by adding a 40 µL sample or control to each hole of an SMA plate and incubating the plate at 33±2°C for 20 hours. Nattokinase (100 g/10 mL) was used as the positive control (K+), and 0.1 M phosphate buffer pH 7.00 was used as the negative control (K-). If there was a clear zone around the hole, it showed proteolytic activity, as shown in **Equation 1**.

$$Proteolytic\ index = \frac{Average\ clear\ zone\ diameter\ (mm)}{Media\ hole\ diameter\ (mm)} \quad [1]$$

Fibrin media preparation and fibrinolytic activity assay

Fibrin plate media was prepared by dissolving 1.5 g agar in 60 mL heated distilled water and pouring 0.5 g fibrin into agar solution. One drop of methylene blue was added to the fibrin medium, then pasteurized for three minutes at 80±2°C. A total of 20 mL was poured into a sterile petri dish using a modified method from Poernomo *et al*.[32]. A fibrinolytic activity assay was done by putting a 40 µL sample or control in each hole of fibrin media on the petri dish and incubating it at 37±2°C for 24 hours. Nattokinase (100 g/10 mL) was used as the positive control, and phosphate buffer 0.1 M pH 7.00 was used as the negative control. The clear zone around the hole indicated fibrinolytic activity, as shown in **Equation 2**.

$$Fibrinolytic\ index = \frac{Average\ clear\ zone\ diameter\ (mm)}{Media\ hole\ diameter\ (mm)} \quad [2]$$

Data analysis

Microsoft Excel (Microsoft Office 2016) was used to calculate the average and standard deviation of the proteolytic and fibrinolytic indexes. Proteolytic and fibrinolytic indexes from the data were analyzed by SPSS statistical software version 25.0 (SPSS Inc., US). Each data analyzed variance at 5% probability to know if there was a significant difference from the index value. The Tukey test was performed at a 5% probability if there was significance.

RESULTS AND DISCUSSION

In this study, the crude enzyme was supernatant obtained from fermented *H. annuus* and *P. vulgaris* seeds extraction by *R. microsporus* var. *oligosporus* FNCC 6010. In the production of enzymes by the SSF method, the fermentation product can be used directly as a source of crude enzymes[33]. Based on the proteolytic activity assay, data were determined that crude enzymes from the fermentation of *H. annuus* and *P. vulgaris* seeds both showed clear zones around the hole (**Figure 2**). Nattokinase as positive control showed the same result, while a 0.1 M phosphate buffer of pH 7.00 as negative control did not show a clear zone. The clear zone showed proteolytic activity per unit time calculated every 24 hours. The higher the protease activity, the higher the clear zone formed[20]. The SMA plate method determined protease activity using casein as substrate[34]. Casein hydrolysis is used to demonstrate the hydrolytic activity of proteases that catalyze casein degradation by breaking the CO-NH peptide bond and releasing amino acids[35].

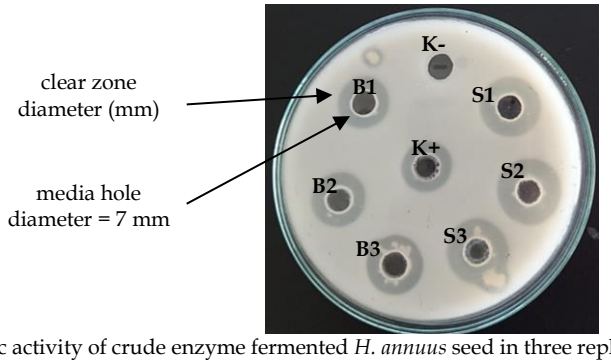

Figure 2. Proteolytic activity of crude enzyme fermented *H. annuus* seed in three replications (S1, S2, and S3) and *P. vulgaris* seed in three replications (B1, B2, and B3) on SMA plate.

Proteases from the fermentation of each seed can be formed during the fermentation process by *R. microsporus* var. oligosporus FNCC 6010. Related research states that the fermentation of soybeans by *R. oligosporus* produces a variety of enzymes, of which one is protease[17]. The enzyme proteases were produced during the fermentation of soybeans by *R. oligosporus* as a source of energy for growth and metabolism through protein hydrolysis. Fermentation degrades cell structures, including proteins, into amino acids and peptides[36].

The recent study revealed that *R. oligosporus* grown on *H. annuus* meal media or seed oil extraction residues produced protease enzymes[28] with a maximum activity of 215.75±2.33 PU/g. Another study stated that the SSF method by *Aspergillus* spp. in French beans substrate has also been found to produce proteases[37] in the highest is 62±9.89 µg/mL.

The proteolytic index value of the fermentation seed can be seen in **Table I**. The proteolytic index of the fermented crude enzyme *H. annuus* seed was higher than that of *P. vulgaris* seed with 2.64±0.01 and 2.23±0.04, respectively, which were statistically shown to differ significantly ($\alpha = 0.05$). This suggests that differences in substrate or medium in the fermentation process produce protease enzymes at different activity levels. Other research explained that the production ability of proteolytic enzymes differs depending on the medium used, where the media degraded by *R. oligosporus* produces different proteolytic enzyme activity[12]. A study of the fermentation of black soybeans had the highest proteolytic index among the five *R. oligosporus* fermented seeds, including komak beans, soybeans, kidney beans, and tholo beans[38]. The difference in the diameter of the clear zone in the SMA plate result supported this finding. Another study showed that *R. oligosporus* grown on various media, wheat bran, rice bran, and soybean media in the SSF method produced proteases with the highest activity of 3.9×10^5. In addition, *R. arrizhus* in whey and cottonseed media produces proteolytic enzymes with a maximum value of up to 170 U/mL.

Table I. Proteolytic index of crude enzyme fermented *H. annuus* and *P. vulgaris* seeds.

Sample / control	Mean clear zone diameter±SD (mm)	Mean proteolytic index±SD
Positive control (K+)	15.17±0.29	2.17±0.04
Negative control (K-)	0.00±0.00	0.00±0.00
Crude enzymes fermented *H. annuus* seed	18.45±0.04	2.64±0.01
Crude enzyme fermented *P. vulgaris* seed	15.62±0.31	2.23±0.04

In this study, crude extract, which is the extraction of unfermented seeds and the subsequent taking of supernatant, and the suspension of *R. microsporus* var. *oligosporus* used for the fermentation process was also tested for proteolytic activity (**Figure 3**) to prove the difference in proteolytic activity between fermented and unfermented seeds or against the suspension of spores used for fermentation. A crude extract from an unfermented *H. annuus* seed showed protease activity at low values (**Figure 3a**). So does the crude extract of *P. vulgaris* seed (**Figure 3b**). The activity is likely to come from the seeds themselves. Previous research has shown that *H. annuus* seed extract produces several enzymes, including protease[27]. *Phaseolus vulgaris* seed is also reported to contain the enzyme protease, so there is a clear zone around the hole[30]. Suspension *R. microsporus* var. *oligosporus* also exhibits proteolytic activity. Related research states that *Rhizopus* and *Mucor* from soil-derived produce fibrinolytic protease enzymes[1]. In addition, proteases can be formed during the culture enrichment process, which involves fungus growth on PDA media. A similar study stated that the suspension of *R. oryzae* had protease activity, even though it was relatively lower than the fermentation results of soybean *tempeh*.

The results showed that the proteolytic activity shown with the mean value of the clear zone and standard deviation in the crude enzyme of fermented seeds was more remarkable compared to the suspension of *R. microsporus* var. *oligosporus* and crude extract of unfermented seeds (**Figure 4**), so do the proteolytic index (**Figure 5**). The ANOVA One-way statistical test stated a significant difference between each proteolytic index with a value of $\alpha = 0.05$, followed by the Tukey HSD posthoc test. From the results of statistical tests, it is concluded that the proteolytic index, from the highest to the lowest, is a crude enzyme from fermented seed; suspension *R. microsporus* var. *oligosporus*, crude extract from the unfermented seed. The proteolytic activity assay is one of the initial tests against fibrinolytic enzymes. Fibrinolytic enzymes belong to the group of proteases that can degrade fibrin 38. For example, one of the proteases derived from *Pergularia extensa* latex has been studied to act similarly to thrombin in releasing fibrinopeptides A and B from fibrinogen acting as thrombolytic agents[3]. Furthermore, the fibrinolytic activity test was carried out using the fibrin plate method to prove the fibrinolytic activity of crude enzymes from fermented *H. annuus* and *P. vulgaris* seeds, which have been proven to have protease activity.

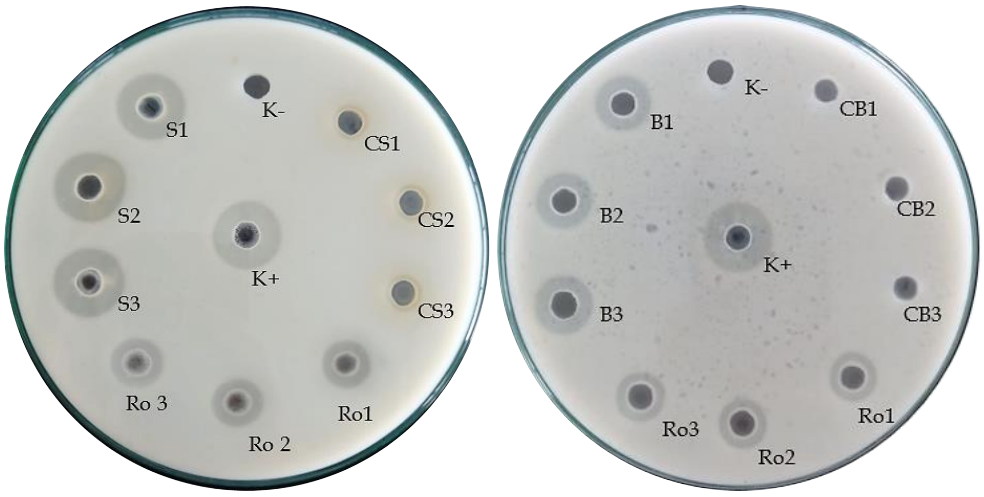

Figure 3. (**a**) Proteolytic activity of crude enzyme from fermented *H. annuus* seed (S1, S2, and S3) and unfermented *H. annuus* seed (CS1, CS2, and CS3); (**b**) Proteolytic activity of crude enzyme from fermented *P. vulgaris* seed (B1, B2, and B3) and unfermented *P. vulgaris* seed (CB1, CB2, and CB3); and suspension of *R. microsporus* var. *oligosporus* FNCC 6010 (Ro1, Ro2, and Ro3).

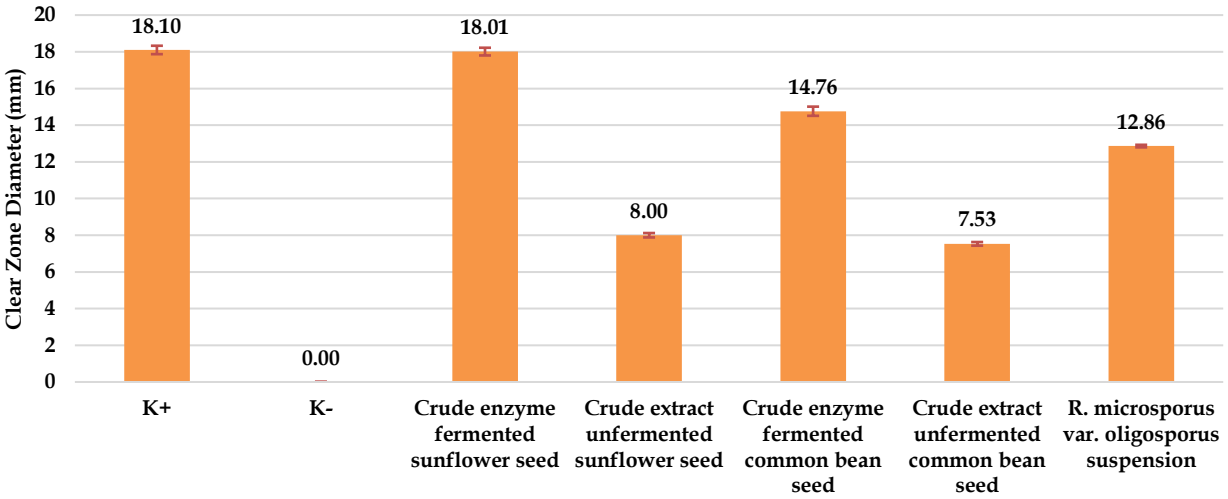

Figure 4. The clear zone diameter of crude enzyme fermented and crude extract unfermented both *H. annuus* and *P. vulgaris* seeds.

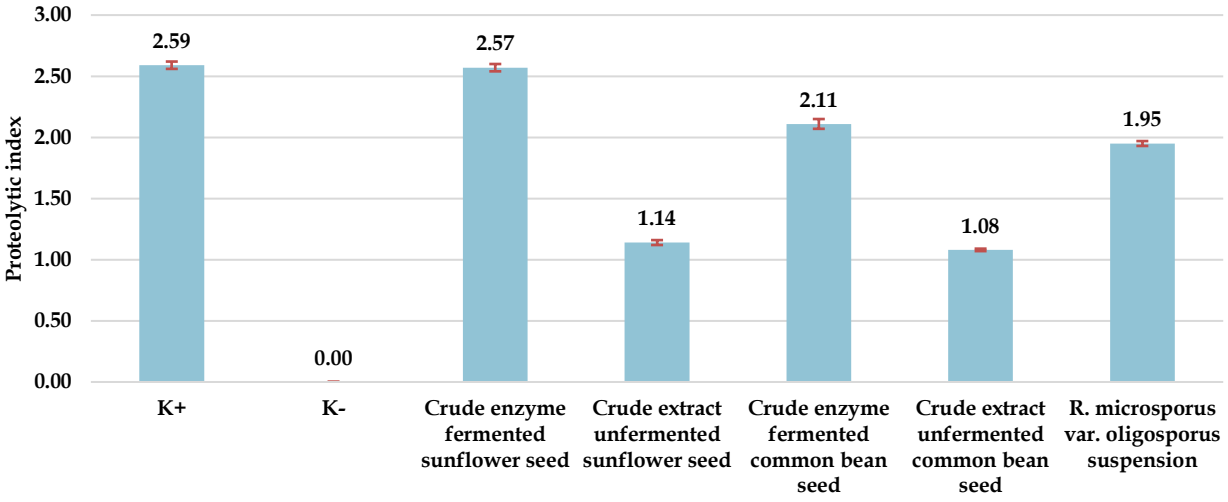

Figure 5. Proteolytic index of crude enzyme fermented and crude extract unfermented both *H. annuus* and *P. vulgaris* seeds.

Based on the fibrinolytic activity assay result, it was known that crude enzymes from the fermented *H. annuus* and *P. vulgaris* seeds by *R. microsporus* var. *oligosporus* produce a clear zone around the well. Nattokinase, as the positive control, gave a similar zone, while a phosphate buffer, as the negative control, did not show a zone (**Figure 6**). The clear zone around the well indicates fibrinolytic activity in the fibrin plate media caused by the hydrolysis of fibrin into soluble amino acids[9,20]. Related research revealed the findings of fibrinolytic enzymes in the fermentation of black soybeans by *R. oligosporus*, which shows *in vitro* fibrinolytic and thrombolytic activity[12]. Another study found that using *H. annuus* oil cake as a growth medium for *Candida guilliermondii* NRRL Y-2075 by the SSF method produced fibrinolytic enzymes[39]. *Bacillus amyloliquefaciens* and *B. subtilis* have grown on agro-industrial substrates, chickpeas, produce enzymes that show thrombolytic activity[40].

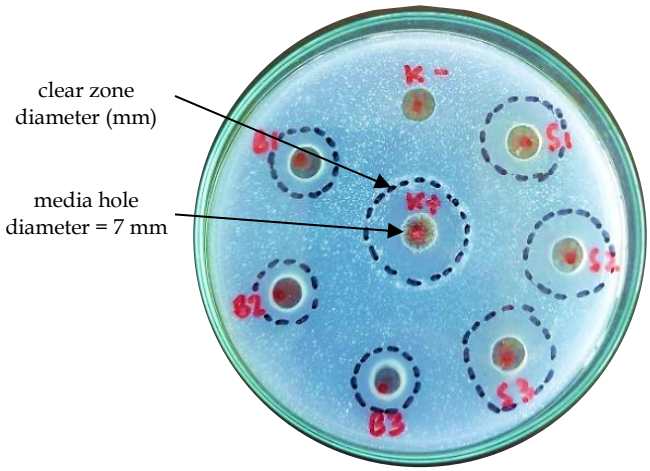

Figure 6. Fibrinolytic activity of crude enzymes fermented *H. annuus* seed in triplicate (S1, S2, and S3) and *P. vulgaris* seed in triplicate (B1, B2, and B3) on fibrin-agarose plates.

The fibrinolytic activity of crude enzymes fermented by *H. annuus* seed was greater than that of *P. vulgaris* seed with a fibrinolytic index of 2.40±0.06 and 1.64±0.06, respectively, significantly different (α=0.05). This is in line with the proteolytic activity test, which means that the fibrinolytic activity is sourced from the protease enzyme. The fibrinolytic index values can be seen in (**Table II**). Fibrinolytic enzymes are inducible enzymes, so the incorporation of protease-rich substances induces the production of enzymes[20]. Present results showed that fibrinolytic enzyme production depends on the substrate used in the SSF. As a substrate of *Bacillus sp*., fermented potato peel demonstrated the highest enzyme activity than corn husk and banana peel[41].

Table II. Fibrinolytic index of crude enzyme fermented *H. annuus* and *P. vulgaris* seeds.

Sample / control	Mean clear zone diameter±SD (mm)	Mean proteolytic index±SD
Positive control (K+)	19.50±0.28	2.79±0.04
Negative control (K-)	0.00±0.00	0.00±0.00
Crude enzymes fermented *H. annuus* seed	16.83±0.43	2.40±0.06
Crude enzyme fermented *P. vulgaris* seed	11.46±0.45	1.64±0.06

CONCLUSION

The crude enzyme derived from fermented *H. annuus* seed and *P. vulgaris* seed by *R. microsporus* in the SSF method was shown to have protease fibrinolytic activity with proteolytic and fibrinolytic indexes of 2.64±0.01; 2.23±0.04, respectively, on SMA media and 2.40±0.06; 1.64±0.06 in fibrin-agarose media. The value is significantly greater (α=0.05) than neither fermented seeds nor spore suspensions. Therefore, the crude enzyme has the potential to be further researched as a candidate for thrombolytic agents. Thrombolytic activity, purification, characterization, and in-depth research are needed so that enzymes can be developed into preparations for the prevention and treatment of CVDs.

ACKNOWLEDGMENT

The authors declare that the research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

AUTHORS' CONTRIBUTION

Conceptualization, R.L., A.T.P., A.S.; Methodology, R.L., A.T.P.; Validation, R.L.; Formal Analysis, R.L.; Investigation, R.L.; Resources, R.L., A.T.P.; Writing - Original Draft, R.L.; Writing - Review & Editing, R.L., A.T.P.; Visualization, R.L.; Supervision, A.T.P., A.S.; Funding Acquisition, R.L., A.T.P. All authors read and approved the final manuscript.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest in this research.

REFERENCES

1. Altaf F, Wu S, Kasim V. Role of Fibrinolytic Enzymes in Anti-Thrombosis Therapy. Front Mol Biosci. 2021;8:680397. doi:10.3389/fmolb.2021.680397

2. Kotb E. Purification and partial characterization of serine fibrinolytic enzyme from Bacillus megaterium KSK-07 isolated from kishk, a traditional Egyptian fermented food. Appl Biochem Microbiol. 2015;51:34-43. doi:10.1134/S000368381501007X

3. Dhillon A, Sharma K, Rajulapati V, Goyal A. 7 - Proteolytic Enzymes. In: Pandey A, Negi S, Soccol CR, editors. Current Developments in Biotechnology and Bioengineering: Production, Isolation and Purification of Industrial Products. Amsterdam: Elsevier; 2017. p. 149-73. doi:10.1016/B978-0-444-63662-1.00007-5

4. Kotb E. Activity assessment of microbial fibrinolytic enzymes. Appl Microbiol Biotechnol. 2013;97(15):6647-65. doi:10.1007/s00253-013-5052-1

5. Sharma C, Osmolovskiy A, Singh R. Microbial fibrinolytic enzymes as anti-thrombotics: Production, characterisation and prodigious biopharmaceutical applications. Pharmaceutics. 2021;13(11):1880. doi:10.3390/pharmaceutics13111880

6. Rajaselvam J, Benit N, Alotaibi SS, Rathi MA, Srigopalram S, Biji GD, et al. In vitro fibrinolytic activity of an enzyme purified from Bacillus amyloliquefaciens strain KJ10 isolated from soybean paste. Saudi J Biol Sci. 2021;28(8):4117-23. doi:10.1016/j.sjbs.2021.04.061

7. Yogesh D, Halami PM. Fibrinolytic enzymes of Bacillus spp.: An overview. Int Food Res J. 2017;24(1):35-47.

8. Stephani L, Tjandrawinata RR, Afifah DN, Lim Y, Ismaya WT, Suhartono MT. Food Origin Fibrinolytic Enzyme with Multiple Actions. HAYATI J Biosci. 2017;24(3):124-30. doi:10.1016/j.hjb.2017.09.003

9. Sada A, Sugianto NE, Poernomo AT. Produksi Enzim Fibrinolitik Tempe oleh Rhizopus oryzae FNCC 6078. Berkala Ilmiah Kimia Farmasi. 2021;8(1):1-6. doi:10.20473/bikfar.v8i1.31202

10. Xiao-Lan L, Lian-Xiang D, Fu-Ping L, Xi-Qun Z, Jing X. Purification and characterization of a novel fibrinolytic enzyme from Rhizopus chinensis 12. Appl Microbiol Biotechnol. 2005;67(2):209-14. doi:10.1007/s00253-004-1846-5

11. Wei X, Luo M, Xu L, Zhang Y, Lin X, Kong P, et al. Production of fibrinolytic enzyme from bacillus amyloliquefaciens by fermentation of chickpeas, with the evaluation of the anticoagulant and antioxidant properties of chickpeas. J Agric Food Chem. 2011;59(8):3957-63. doi:10.1021/jf1049535

12. Poernomo AT, Isnaeni, Purwanto. Thrombolytic Activity of Fibrinolytic Enzyme from Black Soybean Tempeh (Glycine Soja Sieb. Et Zucc) Fermented by Rhizopus Oligosporus FNCC 6010. Res J Pharm Biol Chem Sci. 2017;8(1):1885-96.

13. Sharma KM, Kumar R, Panwar S, Kumar A. Microbial alkaline proteases : Optimization of production parameters and their properties. J Genet Eng Biotechnol. 2017;15(1):115-26. doi:10.1016/j.jgeb.2017.02.001

14. Zhang S, Wang Y, Zhang N, Sun Z, Shi Y, Cao X, et al. Purification and characterisation of a fibrinolytic enzyme from Rhizopus micro sporus var. tuberosus. Food Technol Biotechnol. 2015;53(2):243-8. doi:10.17113/ftb.53.02.15.3874

15. Jennessen J, Schnürer J, Olsson J, Samson RA, Dijksterhuis J. Morphological characteristics of sporangiospores of the tempe fungus Rhizopus oligosporus differentiate it from other taxa of the R. microsporus group. Mycol Res. 2008;112(5):547-63. doi:10.1016/j.mycres.2007.11.006

16. Ahnan-winarno AD, Winarno FG, Gibbons J. Tempeh: A semicentennial review on its health benefits, fermentation, safety, processing, sustainability, and affordability. Compr Rev Food Sci Food Saf. 2021;20(2):1717-67. doi:10.1111/1541-4337.12710

17. Lim J, Nguyen TTH, Pal K, Kang CG, Park C, Kim SW, et al. Phytochemical properties and functional characteristics of wild turmeric (Curcuma aromatica) fermented with Rhizopus oligosporus. Food Chem X. 2021;13:100198. doi:10.1016/j.fochx.2021.100198

18. Nout MJR, Kiers JL. Tempe fermentation, innovation and functionality: update into the third millenium. J Appl Microbiol. 2005;98(4):789-805. doi:10.1111/j.1365-2672.2004.02471.x

19. Srivastava N, Srivastava M, Ramteke W, Mishra K. Chapter 23 - Solid-State Fermentation Strategy for Microbial Metabolites Production: An Overview. In: Gupta VK, Pandey A, editors. New and Future Developments in Microbial Biotechnology and Bioengineering: Microbial Secondary Metabolites Biochemistry and Applications. Amsterdam: Elsevier; 2019. p. 345-54. doi:10.1016/B978-0-444-63504-4.00023-2

20. Ahamed NA, Arif IA, Al-rashed S, Panneerselvam A, Ambikapathy V. In vitro thrombolytic potential of fibrinolytic enzyme from Brevibacterium sp. isolated from the root of the plant, Aloe castellorum. J King Saud Univ Sci. 2022;34(3):101868. doi:10.1016/j.jksus.2022.101868

21. Jo C, Zhang J, Tam JM, Church GM, Khalil AM, Segrè D, et al. Unlocking the magic in mycelium: Using synthetic biology to optimize filamentous fungi for biomanufacturing and sustainability. Mater Today Bio. 2023;19:100560. doi:10.1016/j.mtbio.2023.100560

22. Hermansyah B, Lokapirnasari WP. Pengaruh Subtitusi Tepung Biji Bunga Matahari (Helianthus Annuus L.) dalam Pakan Komersial dengan Konsentrasi Tertentu Terhadap Performa Ayam Pedaging. J Med Vet. 2019;2(1):7-12. doi:10.20473/jmv.vol2.iss1.2019.7-12

23. Lakshmi NJ, Vanaja M, Yadav SK, Maheswari M, Archana G, Patil A, et al. Effect of CO2 on growth, seed yield and nitrogen uptake in sunflower. J Agrometeorol. 2017;19(3):195-9. doi:10.54386/jam.v19i3.620

24. Franco R, Iseppi L, Taverna M. Sunflower Oil Functional Properties for Specialty Food. Nutr Food Sci Int J. 2018;5(4):4-7. doi:10.19080/nfsij.2018.05.555668

25. Karefyllakis D, van der Goot AJ, Nikiforidis CV. Multicomponent emulsifiers from sunflower seeds. Curr Opin Food Sci. 2019;29:35-41. doi:10.1016/j.cofs.2019.07.005

26. Grasso S, Pintado T, Pérez-Jiménez J, Ruiz-Capillas C, Herrero AM. Potential of a sunflower seed by-product as animal fat replacer in healthier frankfurters. Foods. 2020;9(4):445. doi:10.3390/foods9040445

27. Özacar M, Mehde AA, Mehdi WA, Özacar ZZ, Severgün O. The novel multi cross-linked enzyme aggregates of protease, lipase, and catalase production from the sunflower seeds, characterization and application. Colloids Surf B Biointerfaces. 2019;173:58-68. doi:10.1016/j.colsurfb.2018.09.042

28. Rauf A, Irfan M, Nadeem M, Ahmed I, Iqbal HMN. Optimization of Growth Conditions for Acidic Protease Production from Rhizopus oligosporus through Solid State Fermentation of Sunflower Meal. Int J Biotechnol Bioeng. 2010;4(12):898-901. doi:10.5281/zenodo.1079814

29. Celmeli T, Sari H. The Nutritional Content of Common Bean (Phaseolus vulgaris L.) Landraces in Comparison to Modern Varieties. Agronomy. 2018;8(9):166. doi:10.3390/agronomy8090166

30. Rodríguez L, Mendez D, Montecino H, Carrasco B, Arevalo B, Palomo I, et al. Role of Phaseolus vulgaris L. in the Prevention of Cardiovascular Diseases-Cardioprotective Potential of Bioactive Compounds. Plants. 2022;11(2):186. doi:10.3390/plants11020186

31. Chávez-Mendoza C, Hernández-Figueroa KI, Sánchez E. Antioxidant capacity and phytonutrient content in the seed coat and cotyledon of common beans (Phaseolus vulgaris L.) from various regions in Mexico. Antioxidants. 2019;8(1):5. doi:10.3390/antiox8010005

32. Poernomo AT, Isnaeni, Purwanto. Aktivitas Invitro Enzim Fibrinolitik Ekstrak Tempe Hasil Fermentasi Rhizopus Oligosporus ATCC 6010 Pada Substrat Kedelai Hitam. Berkala Ilmiah Kimia Farmasi. 2015;4(2):19-26.

33. Sambo S, Magashi AM, Farouq AA, Hassan SW. An overview of the solid state fermentation in the production of fungal protease enzymes. World J Adv Res Rev. 2021;9(3):85-9. doi:10.30574/wjarr.2021.9.3.0061

34. Susanti E, Lutfiana N, Suharti, Retnosari R. Screening of proteolytic bacteria from tauco Surabaya based on pathogenicity and selectivity of its protease on milky fish (Chanos chanos) scales for healthy and halal collagen production. IOP Conf Ser Mater Sci Eng. 2019;509:012044. doi:10.1088/1757-899X/509/1/012044

35. Muisristanto D, Poernomo AT, Sugijanto. Isolasi dan Penapisan Fibrinolitik Jamur Tanah Hutan Mangrove Wonorejo Surabaya. Berkala Ilmiah Kimia Farmasi. 2015;4(2):11-7.

36. Handoyo T, Morita N. Structural and Functional Properties of Fermented Soybean (Tempeh) by Using Rhizopus oligosporus. Int J Food Prop. 2006;9(2)347-55. doi:10.1080/10942910500224746

37. Upgade A, Nandeshwar A, Samant L. Assessment of fungal protease enzyme from French bean using A. niger by Solid State Fermentation. J Microbiol Biotechnol Res. 2011;1(4):45-51.

38. Raju EVN, Divakar G. An Overview on microbial fibrinolytic proteases. Int J Pharm Sci Res. 2014;5(3):643-56. doi:10.13040/IJPSR.0975-8232.5(3).643-56

39. Rashad MM, Mahmoud AE, Al-Kashef AS, Nooman MU. Purification and characterization of a novel fibrinolytic enzyme by Candida guilliermondii grown on sunflower oil cake. J Appl Sci Res. 2012;8(2):635-45.

40. Akhtar T, Hoq M, Mazid A. Bacterial Proteases as Thrombolytics and Fibrinolytics. Dhaka Univ J Pharm Sci. 2017;16(2):255-69. doi:10.3329/dujps.v16i2.35265

41. Gond SP, Sharma RA. Agro Waste Mediated Production of Fibrinolytic Enzyme and Its Optimization and Application. Int J Sci Res. 2022;11(2):58-63. doi:10.21275/SR22130105418