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Trypsin: A Member of The Serine Protease Enzyme Isolated from Fusarium oxysporum and Fusarium sp.

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Abstract

Trypsin/Porcine enzyme is widely used in the vaccine manufacturing industry, but due to its non-halal source (intestine of Pig Sus scrofa), it needs an alternative enzyme that has the same characteristics but is sourced from halal things. Fusarium sp. and Fusarium oxysporum are thought to have the ability to produce protease enzymes like Trypsin from pigs, which is a natural part of their infection system to cultivated plants. This study was conducted to isolate and characterize enzymes isolated from Fusarium oxysporum and Fusarium sp. Enzyme isolation was carried out using ammonium sulfate precipitation technique. Enzyme characterization using uv-vis spectrophotometer method by making variations of pH, temperature and type of substrate (specific gravity of substrate). The results suggest that both novel trypsin enzymes, derived from Fusarium oxysporum and Fusarium sp., exhibit similar optimal conditions for activity, with an optimum pH of around 7-7.5 and an optimum temperature of 30°C. However, there are differences in their kinetic parameters: the trypsin from Fusarium oxysporum has a higher Vmax (8.183 mM/s) and Km (0.9091 mM) compared to the trypsin from Fusarium sp., which has a Vmax of 6.911 mM/s and a lower Km (0.6192 mM). These findings indicate that the enzyme from Fusarium oxysporum may have a higher catalytic efficiency, while the enzyme from Fusarium sp. may exhibit higher substrate affinity.

1. INTRODUCTION

Enzymes play a crucial role in various biological mechanisms, including as essential compounds needed in cellular metabolism processes. Among the various enzymes involved in biological processes, trypsin, a serine protease enzyme, has been widely

utilized in biotechnology, particularly in protein degradation [1].

The utilization of protein degradation mechanisms, particularly using the serine protease enzyme trypsin, is prevalent in vaccine production industries. The production of rotavirus vaccines utilizes trypsin enzymes derived from pig intestines, known as porcine trypsin. Vaccines such as MMR, Singles, and Flu all utilize porcine trypsin as a stabilizer to maintain vaccine efficacy and safety during storage [2]. However, the use of enzymes derived from pigs poses challenges in countries with large Muslim populations due to religious dietary restrictions. Therefore, there is a need for alternative trypsin enzymes sourced from halal-certified sources.

Research on protease enzymes sourced from microorganisms is currently extensive due to their significant potential in pharmaceuticals, food, and biotechnology industries. Fusarium, a genus of fungi, exhibits pathogenic properties in cultivated crops such as maize and other grains. Fusarium's pathogenic mechanism involves producing serine protease enzymes capable of cleaving peptide bonds within the cell walls of cultivated plants. By disrupting the integrity of the plant cell walls through peptide bond cleavage, Fusarium creates entry points for invading plant cells. This process facilitates fungal penetration and spread within plant tissues [3].

The pathogenic mechanism of Fusarium towards cultivated crops by producing serine protease enzymes can be adapted for vaccine production processes. Serine proteases serve several functions in vaccine manufacturing, including cleaving and processing viral or bacterial proteins, cleaving fusion proteins or tags added to aid protein expression and purification, activating viral proteins during replication, and generating specific protein fragments that act as antigens to stimulate immune responses [4]. Therefore, there is a need to identify novel trypsin enzymes produced by *Fusarium sp.* and *Fusarium oxysporum* for degrading gelatin substrates as an alternative to porcine trypsin.

2. MATERIALS AND METHODS

Inoculation of Fusarium on Selective Media

Subcultures of *Fusarium sp.* and *Fusarium oxysporum* were maintained on PDA media as research stock. To assess the activity of the produced trypsin enzyme, Fusarium isolates were inoculated on solid gelatin selective media using the streak plate method. The growth was observed over a 48-hour period with observations recorded at 12, 24, and 48 hours.

Enzyme Extraction

Enzyme extraction was performed using a specific method. The novel enzyme was extracted by cultivating in liquid media supplemented with ammonium sulfate ($(NH_4)_2SO_4$), followed by homogenization at low temperatures for 24 hours. After a 24-hour incubation, the crude extract was centrifuged at 7000 rpm for 15 minutes to obtain a semi-purified enzyme. The semi-purified enzyme was then dialyzed using a cellulose membrane at pH 7 and immersed in 0.025 phosphate buffer to obtain the pure enzyme.

Characterization of Trypsin Enzyme

The study characterized the novel trypsin enzyme from Fusarium in terms of optimal pH, temperature, and substrate

1. **Optimal pH**

Prepared using liquid gelatin media with added NaOH and KH2PO4 buffers. Each media variation was supplemented with 1 ml of the novel trypsin enzyme and 2.5 ml of 5% TCA, then incubated for 30 minutes at room temperature. The incubated enzyme and substrate were then centrifuged at 400 rpm for 20 minutes, and 1 ml of the filtrate was collected. Absorbance values were measured using a UV-Vis spectrophotometer at λ = 275 nm.

2. Optimal Temperature

The optimal temperature was identified at variations of 25, 30, 37, 40, 45, and 50°C using 0.5% liquid gelatin media, supplemented with 2.5 ml TCA and 1 ml of the pure enzyme. After a 30-minute incubation, the media and substrate mixture were centrifuged at 400 rpm for 20 minutes. Subsequently, 1 ml of the filtrate was collected, and absorbance values were measured using a UV-Vis spectrophotometer at λ = 275 nm.

3. Optimal Substrate

The optimal substrate was identified using azogelatin substrate variations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.65%, with the addition of 1 ml of the pure enzyme and 2.5 ml TCA. The substrate and enzyme mixture were incubated for 30 minutes at the optimal temperature and pH determined from previous procedures, then centrifuged at 400 rpm for 20 minutes. Afterward, 1 ml of the filtrate was collected, and absorbance values were measured using a UV-Vis spectrophotometer at λ = 275 nm.

Data analysis

Data analysis was performed by describing the quantitative data obtained from the tests conducted. To determine the optimal substrate, the Vmax and Km values (Michaelis-Menten constant) were calculated by creating a Lineweaver-Burk plot from the enzyme reaction data across various substrate concentrations

3. RESULTS and DISCUSSION

Pure cultures of *Fusarium sp.* and *Fusarium oxysporum* were obtained on solid gelatin media. Observations of the pure cultures at 24 hours, 48 hours, 72 hours, and 10 days revealed the following macroscopic characteristics of *Fusarium sp.*: the colony diameter at 24 hours was 10.34 mm, at 48 hours was 10.82 mm, at 72 hours was 11.31 mm, and at 10 days was 32.18 mm. For *Fusarium oxysporum*, the colony diameter at 24 hours was 6.23 mm, at 48 hours was 6.52 mm, at 72 hours was 6.82 mm, and at 10 days was 53.82 mm. Both *Fusarium sp.* and *Fusarium oxysporum* exhibited robust growth and dense hyphal proliferation. The extensive hyphal growth in *Fusarium sp.* indicates that the fungi can thrive on gelatin media, suggesting their ability to break nitrogen bonds with the aid of trypsin enzymes (Table 1).

The dialysis method produced semi-purified enzymes characterized by darker, more concentrated precipitates in *Fusarium oxysporum* and lighter precipitates in *Fusarium sp.* The pure enzyme from *Fusarium oxysporum* appeared dark brown but clear, while the enzyme from *Fusarium sp.* was pale yellow-white but concentrated. Using acetone, both *Fusarium oxysporum* and *Fusarium sp.* enzymes appeared clear (Figure 1).

After successful enzyme extraction, enzyme activity was analyzed to determine the optimal pH, temperature, and substrate amount for reaction with the novel trypsin enzyme. Spectrophotometric observations of pH variations showed that the novel trypsin enzyme from *Fusarium oxysporum* exhibited the best catalytic activity at pH 7, while the novel trypsin enzyme from *Fusarium sp.* showed optimal activity at pH 6. Additionally, optimal temperature and maximum substrate concentration analyses were performed. The optimal temperature for both novel trypsin enzymes from *Fusarium oxysporum* and *Fusarium sp.* was 30°C (Figure 2).

Using the determined optimal pH and temperature, substrate variation analysis was conducted with the novel enzyme. From this test (Table 2), the Vmax and Km values were obtained: Vmax 8.183 mM/s and Km 0.9091 mM for the novel trypsin enzyme from *Fusarium oxysporum*, and Vmax 6.911 mM/s and Km 0.6192 mM for the novel trypsin enzyme from *Fusarium sp.* (Figure 3).

Table 1. Effect of liquid organic fertilizer, inorganic fertilizer, and their combination on plant height, number of leaves, and leaf area of peanut plants

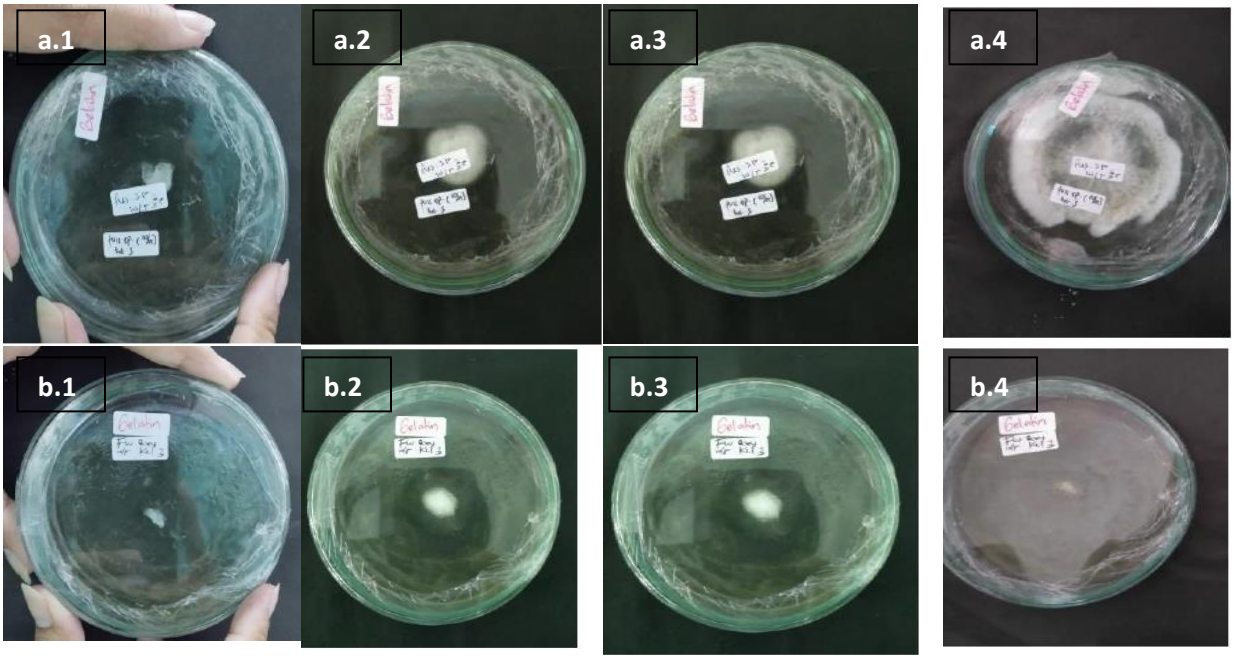

Figure 1. The results of fungal growth in gelatin media. *Fusarium sp.* (a) and *Fusarium oxysporum* (b) on gelatin media with incubation periods of 12 hours (1), 24 hours (2), 48 hours (3), and 10 days (4).

Table 1. The Diameters of Fungal Growth in Gelatin Media.

No.	Isolate	12 (hours)	24 (hours)	48 (hours)	10 (days)
1.	*Fusarium oxysporum*	12,1 mm	26,67 mm	26,67 mm	67,94 mm
2.	*Fusarium sp.*	7,46 mm	11,42 mm	11,42 mm	41,40 mm

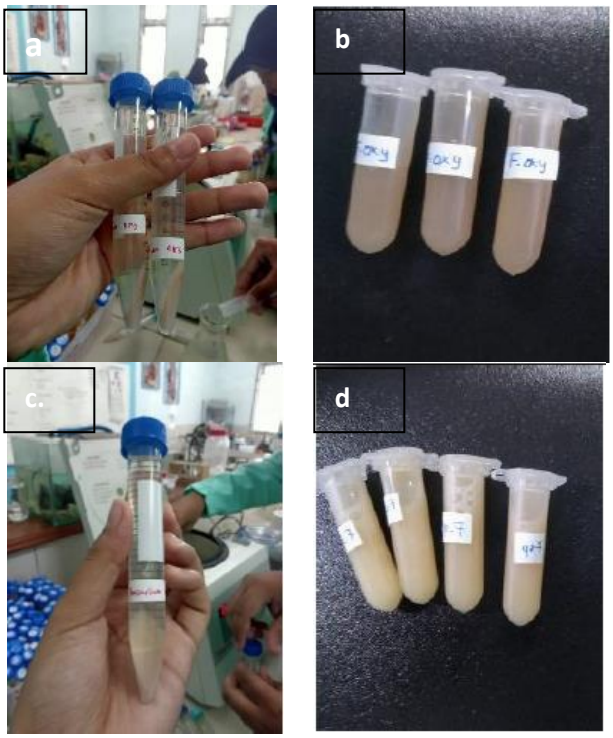

Figure 2. Results of the extraction novel enzyme trypsin from (**a**) *Fusarium oxysporum* (crude extract), (**b**) *Fusarium oxysporum* (pure extract), (**c**) *Fusarium sp.* (crude extract), and (**d**) *Fusarium sp.* (pure extract).

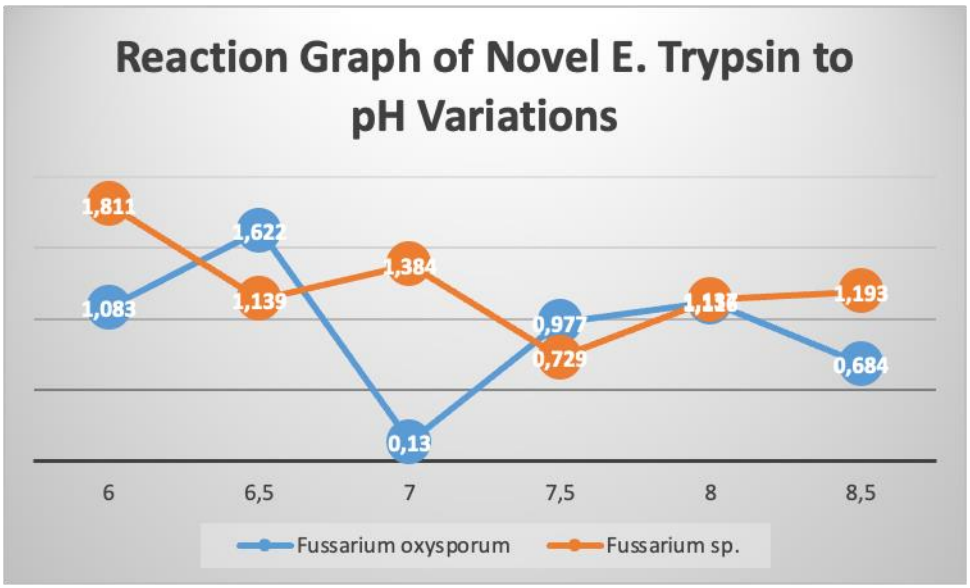

Figure 3. Results of the optimum pH analysis for the reaction of the novel enzyme trypsin from *Fusarium oxysporum* and *Fusarium sp.* isolates based on their absorbance values at λ = 275 nm

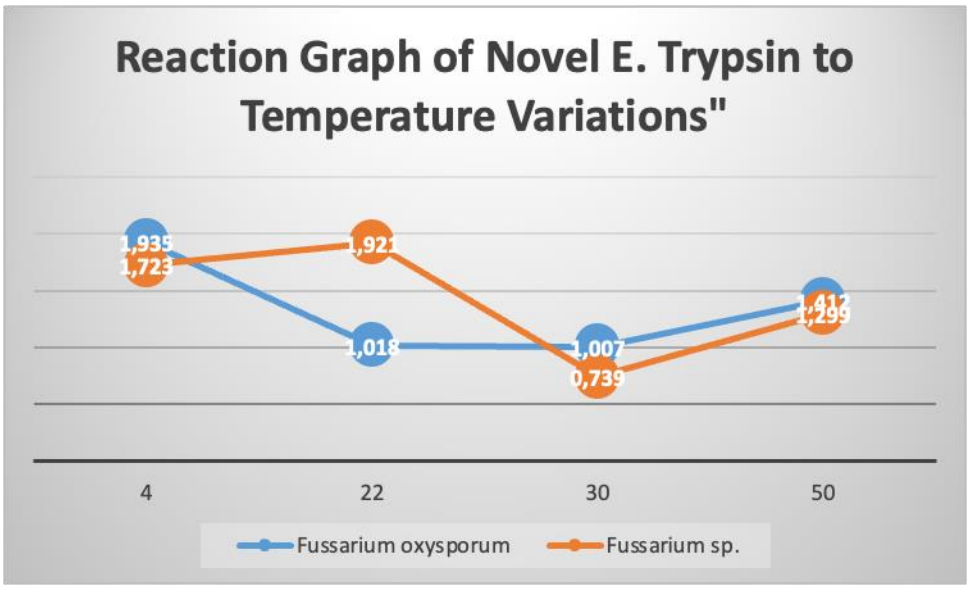

Figure 4. Results of the optimum temperature analysis for the reaction of the novel enzyme trypsin from *Fusarium oxysporum* and *Fusarium sp.* isolates based on their absorbance values at λ = 275 nm

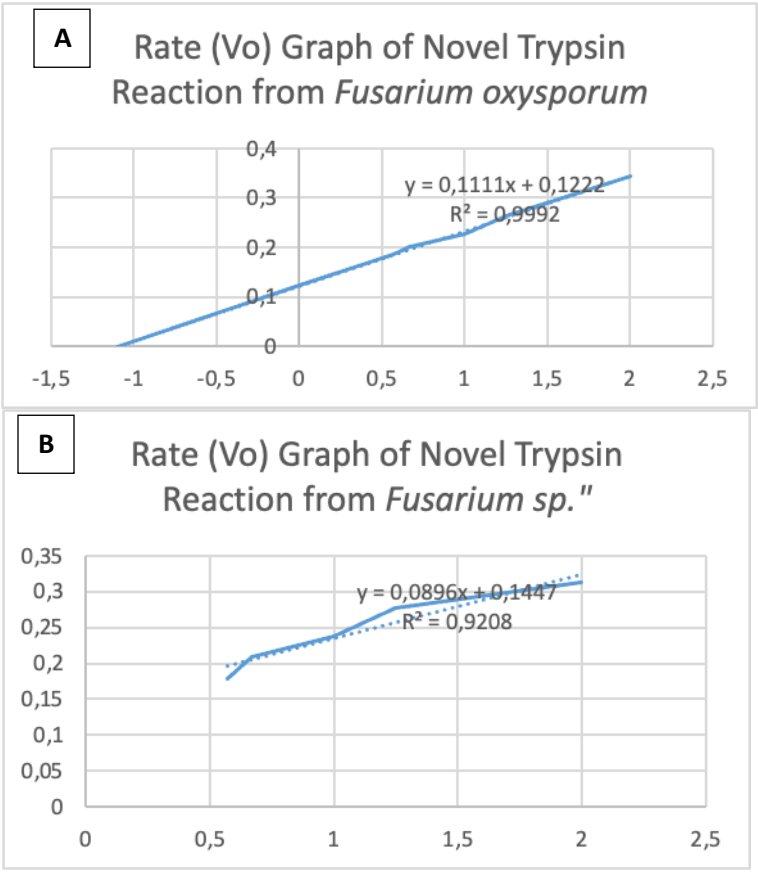

Figure 5. Linear Lineweaver-Burk plot for the analysis of Vmax and Km of the novel enzyme trypsin from (A) *Fusarium oxysporum* and (B) *Fusarium sp.* isolates

4. DISCUSSION

Pure cultures of *Fusarium sp.* and *Fusarium oxysporum* were obtained on solid gelatin media. Observations of the pure cultures at 24 hours, 48 hours, 72 hours, and 10 days revealed the following macroscopic characteristics of *Fusarium sp.*: the colony diameter at 24 hours was 10.34 mm, at 48 hours was 10.82 mm, at 72 hours was 11.31 mm, and at 10 days was 32.18 mm. For *Fusarium oxysporum*, the colony diameter at 24 hours was 6.23 mm, at 48 hours was 6.52 mm, at 72 hours was 6.82 mm, and at 10 days was 53.82 mm. Both *Fusarium sp.* and *Fusarium oxysporum* exhibited robust growth and dense hyphal proliferation. The extensive hyphal growth in *Fusarium sp.* indicates that the fungi can thrive on gelatin media, suggesting their ability to break nitrogen bonds with the aid of trypsin enzymes (Table 1).

The conditions of isolates between *Fusarium sp.* and *Fusarium oxysporum* grown on solid gelatin media show distinct differences. The isolate condition of *Fusarium sp.* in solid gelatin media incubated for a longer period (10 days) showed contamination in the media, hindering hyphal growth. In contrast, the isolate *Fusarium oxysporum* remained uncontaminated from day 1 to day 10. Both isolates also displayed differences in colony diameter, with *Fusarium sp.* showing better survival in solid gelatin media compared to *Fusarium oxysporum*. This aligns with [5], who noted that *Fusarium sp.* can survive better when environmental conditions are favorable. Additionally, *Fusarium sp.* exhibited faster hyphal spread in solid gelatin media, resulting in a larger colony diameter than *Fusarium*

oxysporum. This is consistent with [6], who stated that good nutrient availability in the culture media enhances the survival and hyphal spread of *Fusarium* sp.

The ability of the isolate to survive in gelatin media indicates its capacity to produce enzymes that can degrade gelatin, one of which is the novel enzyme trypsin. The mechanism by which trypsin degrades gelatin involves the hydrolysis of peptide bonds within the gelatin protein matrix. Trypsin specifically targets peptide bonds where the carbonyl group is contributed by lysine or arginine. When gelatin, a denatured form of collagen, is introduced, trypsin binds to these specific sites. The active site of trypsin contains a catalytic triad composed of histidine, aspartate, and serine, which facilitate the hydrolysis reaction. The serine residue acts as a nucleophile, attacking the carbonyl carbon of the peptide bond in gelatin, forming a tetrahedral intermediate stabilized by the oxyanion hole. The intermediate then collapses, leading to peptide bond cleavage and the formation of an acyl-enzyme intermediate. A water molecule, activated by histidine, attacks this intermediate, releasing the N-terminal fragment and regenerating the free enzyme, which can catalyze further reactions. This process repeats, progressively breaking down the gelatin into smaller peptides and amino acids.

Ammonium sulfate precipitation is an initial method for enzyme purification, chosen for its high solubility, which facilitates interaction with water molecules [7]. The purification process involves mixing the crude extract with ammonium sulfate, followed by centrifugation to form pellets and supernatant, resulting in a semi-purified enzyme. To obtain a pure enzyme, the pellets are resuspended in phosphate buffer and dialyzed using a cellulose membrane, with the buffer changed every 6 hours. According to [8], this process involves precipitation with ammonium sulfate and dialysis (diffusion using a cellulose membrane). The principle of dialysis is diffusion with a permeable cellulose layer.

The dialysis method produced semi-purified enzymes characterized by darker, more concentrated precipitates in *Fusarium oxysporum* and lighter precipitates in *Fusarium sp.* The pure enzyme from *Fusarium oxysporum* appeared dark brown but clear, while the enzyme from *Fusarium sp.* was pale yellow-white but concentrated. Using acetone, both *Fusarium oxysporum* and *Fusarium sp.* enzymes appeared clear (Figure 1).

The extraction of novel trypsin enzymes from *Fusarium oxysporum* and *Fusarium sp.* shows distinct physical characteristics that can be associated with their enzyme production levels. According to Keilin & Mann (1938), the darker color of the *Fusarium oxysporum* enzyme extract indicates a higher enzyme concentration, suggesting that *Fusarium oxysporum* produces more trypsin compared to *Fusarium sp.*, which has a lighter and denser extract. This observation aligns with the higher Vmax value of *Fusarium oxysporum* (8.183 mM/s), indicating more efficient enzyme activity and possibly higher enzyme production.

The differences in kinetic parameters (Vmax and Km) further support this hypothesis. *Fusarium oxysporum* has a higher Vmax, meaning it can catalyze more substrate per unit time, and a higher Km, suggesting a lower affinity for the substrate compared to *Fusarium sp.*, which has a lower Km (indicating a higher affinity for the substrate). These findings suggest that while both enzymes have similar optimal conditions (pH 7-7.5 and 30°C), *Fusarium oxysporum* produces a more efficient enzyme with higher catalytic activity, consistent with the darker color of its extract.

Furthermore, the purification process using ammonium sulfate in this study is consistent with typical protein purification methods, where salting out aids in isolating the enzyme by reducing its solubility in high salt concentrations. This method could contribute to the higher enzyme concentration and activity observed in the *Fusarium oxysporum* extract, reinforcing the idea that this strain produces more trypsin than

Fusarium sp..After successful enzyme extraction, enzyme activity was analyzed to determine the optimal pH, temperature, and substrate amount for reaction with the novel trypsin enzyme. Spectrophotometric observations of pH variations showed that the novel trypsin enzyme from *Fusarium oxysporum* exhibited the best catalytic activity at pH 7, while the novel trypsin enzyme from *Fusarium sp.* showed optimal activity at pH 6. Additionally, optimal temperature and maximum substrate concentration analyses were performed. The optimal temperature for both novel trypsin enzymes from *Fusarium oxysporum* and *Fusarium sp.* was 30°C (Figure 2).

Using the determined optimal pH and temperature, substrate variation analysis was conducted with the novel enzyme. From this test (Table 2), the Vmax and Km values were obtained: Vmax 8.183 mM/s and Km 0.9091 mM for the novel trypsin enzyme from *Fusarium oxysporum*, and Vmax 6.911 mM/s and Km 0.6192 mM for the novel trypsin enzyme from *Fusarium sp.* (Figure 3).

UV-Vis spectrophotometry observations on the effect of temperature showed different results. For *Fusarium oxysporum*, at 4°C, the value was 1.935, at 22°C, it was 1.018, at 30°C, it was 1.007, and at 50°C, it was 1.412. For *Fusarium sp.*, at 4°C, the value was 1.723, at 22°C, it was 1.921, at 30°C, it was 0.739, and at 50°C, it was 1.299. Pawar et al. (2023)[10] noted that trypsin from Fusarium is a serine protease with an optimal alkaline pH around 10. *Fusarium sp.* has an optimal pH of 9.5 and an optimal temperature of 50°C [11] (Ueda et al., 2007). Hemmati et al. (2017) [12] explained that at pH 10, trypsin activity can reach 70%. The optimal pH varies with the isolate used, affecting the enzyme's protein structure and activity.

The *Fusarium oxysporum* enzyme has a higher Km (0.9091 mM) and a higher Vmax (8.183 mM/s), suggesting that it has a lower affinity for the substrate (higher Km) but can catalyze reactions at a higher maximum rate (higher Vmax) than *Fusarium sp.* The *Fusarium sp.* enzyme has a lower Km (0.6192 mM) and a lower Vmax (6.911 mM/s), indicating a higher affinity for the substrate but a lower overall catalytic capacity.

The Lineweaver-Burk equation is used to calculate the Km and Vmax based on the inverse of the reaction rate (1/v) and the inverse of the substrate concentration (1/[S]). The slope of the Lineweaver-Burk plot gives the value of Km/Vmax, and the y-intercept gives 1/Vmax, while the x-intercept gives -1/Km. Statistically, a comparison of the Lineweaver-Burk plots for both enzymes would show the difference in slope and intercept values. The steeper slope (higher Km/Vmax ratio) for *Fusarium oxysporum* indicates a lower affinity for the substrate, while the flatter slope for *Fusarium sp.* suggests a higher affinity.

The higher Km and Vmax of *Fusarium oxysporum* indicate it could be more suitable for industrial or research applications where higher reaction rates are desired, despite its lower substrate affinity. Conversely, the higher substrate affinity of *Fusarium sp.* (lower Km) suggests it may be more efficient at lower substrate concentrations, potentially making it more suitable for applications where substrate availability is limited.

As protease enzymes (EC 3.4) catalyze the breakdown of proteins, the higher Vmax of *Fusarium oxysporum* suggests it may be more efficient in degrading proteins into smaller peptides or amino acids. These proteases also regulate enzymatic cascades, and *Fusarium oxysporum* might have broader applications in metabolic processes, such as fat and carbohydrate breakdown, due to its higher catalytic efficiency [13].

5. CONCLUSION

catalytic efficiency with a Vmax of 8.183 mM/s and a Km of 0.9091 mM, suggesting it is more effective in catalyzing reactions, particularly in industrial or biotechnological applications requiring high enzyme activity. In

contrast, the *Fusarium sp.* enzyme, with a Vmax of 6.911 mM/s and a lower Km of 0.6192 mM, indicates a stronger substrate affinity, which could be advantageous for processes where the enzyme operates at lower substrate concentrations, potentially enhancing efficiency in more sensitive biochemical applications.

These findings highlight the potential for tailoring enzyme usage based on specific requirements. The *Fusarium oxysporum* enzyme's higher Vmax makes it a promising candidate for applications such as protein degradation in industrial processes or metabolic regulation in biocatalysis, where high throughput is essential. Meanwhile, the *Fusarium sp.* enzyme, with its lower Km, could be explored for more specialized applications in which substrate availability is limited or where higher specificity is required, such as in clinical or therapeutic settings targeting specific peptides or proteins.

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