Efficacy the Ultraviolet Radiation for Enhancing The Yeast's Ability to Produce The L-Asparaginase With Its Isolation and Purification

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ABSTRACT

The current study test the ability of the following yeast strains: *Naganishia uzbekistanensis* M1, *Pichia nakasei* M29, *Moesziomyces aphidis* M36, *Pichia kudriavzevil* M64, *Hanseniaspora guilliermondii* M78, *Hanseniaspora uvarum* M85, *Rhodotorula mucilaginosa* M92 and *Meyerozyma guilliermondii* M120 to produce the L-Asparaginase (ASNase) enzyme with the efficancy ultraviolet (UV) radiation at (2, 5, 10, 15, 20) minutes in stimulating its production. The results showed that all isolates were unable to produce the enzyme except *M. guilliermondii* M120, which demonstrated a high production capability indicated by the appearance of a pink halo resulted from the enzymatic breakdown of the substrate (the amino acid L-Asparagine). UV radiation exposure enhanced *P. kudriavzevil M64*, *H. uvarum M85* and *R. mucilaginosa* M92 to produce the enzyme. The heigh production capability was by *M. guilliermondii* M120 with enzymatic activity of 1.5 units/mL, compared to 1 unit/mL for the non-mutated isolate. Bradford's method for protein concentration estimation revealed an increase in protein concentration in the same yeast isolate from 0.32 mg/mL to 0.45 mg/mL after UV-induced mutation. The ASNase enzyme was isolated and

from *M. guilliermondii* M120 in powder form.

Keywords: *Meyerozyma guilliermondii* M120, UV, Mutation, L-Asparaginase

Introduction

L-Asparaginase is an enzyme that catalyses the hydrolysis of the aminoacid asparagine into aspartic acids and ammonia. This enzyme is ideal for the treatment of some kinds of cancer especially Acute Lymphoblastic Leukemia (ALL). As for the therapeutic effect, it is linked to the elimination of asparagine – an amino acid necessary for the development of cancer cells and their death. (Tsegaye *et al.*, 2024).

The enzyme is produced by a diverse range of organisms including bacteria, fungi and yeasts.(Ramadan *et al.*,2022) Bacteria such as *Escherichia coli* and

Erwinia chrysanthemi are the primary sources of this enzyme in medical applications(Khuder and Mohammed, 2025) However, enzymes produced by bacteria may cause adverse effects such as hypersensitivity and the development of antibodies. Consequently, alternative sources such as fungi and yeasts have been explored to produce enzymes that are more compatible with the human system, thereby reducing side effects (Garcia *et al*., 2023).

According to the research done by Garcia et al. in 2023, it was evident that the filamentous fungi L-Asparaginase including Aspergillus niger and Rhizopus oryzae had better characteristics than bacteria enzymes, effectively reducing the side effects of bacterial produced L-Asparaginase in the human system. Furthermore, a new strain of Rhizopus oryzae (AM16) was identified with higher productivity of L–Asparaginase using wheat bran under solid state fermentation condition. As the anticancer activity of the enzyme was proved effective against several cell lines, the enzyme has potentials as an anticancer drug. (Othman *et al*., 2022; Mohammed and Masyab, 2020).

L-Asparaginase is widely used in medical field, especially for the treatment of lymphomas (Allen *et al*., 2024). It is also used in the food industry to minimize the generation of acrylamide which is likely to cause cancer when starchy foods are fried. The enzyme helps to minimize the high levels of acrylamide that are produced when food is cooked by breaking free asparagine. (Jana *et al*., 2024).

This study aims to enhance productivity of L-Asparaginase by ultraviolet radiation as a means of induction or biological modification in yeasts.

Materials and Methods

Source of Yeast Isolates Used in the Study:

The yeast isolates (*Naganishia uzbekistanensis* M1, *Pichia nakasei* M29, *Moesziomyces aphidis* M36, *Pichia kudriavzevil* M64, *Hanseniaspora guilliermondii* M78, *Hanseniaspora uvarum* M85, *Rhodotorula mucilaginosa* M92 and *Meyerozyma guilliermondii* M120 These isolates were molecularly characterized using conventional PCR based genetic sequencing methodology as described (Falih *et al*., 2025).

Efficiency of Short-Wavelength Ultraviolet Mutagenesis in Producing L-Asparaginase

Samples of yeast cells were irradiated at 254 nm wavelength in a dark chamber using a UV lamp in this study as described by the method (Gao *et al*., 2010). The yeast cells were first inoculated in Sabouraud's Glucose Agar (SGA) medium through a streaking technique, then incubated at 28°C for 24-48 hours before making a 5.0 mL suspension. After that, the plates were supplemented with 5.0

mL of Tween 80 solution. The Tween 80 solution was prepared by adding 1.0 g of Tween 80 to 99 ml of sterilized distilled water by autoclaving the solution. Cells were taken from the plate to get a yeast suspension for using in mutagenesis tests as mentioned by Li *et al* in their study in 2007.

An additional 5 mL aliquot of the suspension was taken and pipetted onto a clean, sterile Petri dish and the dish placed on a Gallen Kamp magnetic stirrer, UK, to ensure that all the cells were equally exposed to the UV rays. The dish cover was removed, and the suspension was irradiated vertically, maintaining a distance of 20 cm between the lamp and the dish surface (Li *et al*., 2007). The irradiation process was performed for each isolate across five time intervals: 0, 2, 5, 10, 15, and 20 minutes. After irradiation, the dish was wrapped in aluminum foil and left in the dark for an hour to prevent photoreactivation.

The suspension was mixed well and five SGA plates were inoculated using the spread plate method by adding 0.2mL of the irradiated yeast suspension per time interval. The plates were then incubated at 28°C for one week and the number of colony was counted. The total number of colonies in 1.0 mL of the irradiated suspension for each time interval was compared with the non-irradiated suspension. A series of dilutions up to 10^4 was prepared, and ten SGA plates were inoculated with 0.1 mL of the diluted suspension per plate, incubated at 28°C for seven days.

Testing the Ability of Isolates to Produce L-Asparaginase (ASNase)
For studing the ability of isolates to produce L-asparaginase, McDox agar (Ranjini Priya and Subhashini, 2022) was prepared, then sterilizated with coolled ato room temperature, was supplemented with 0.08 M L-asparagine (10 g/L) and 0.3 mL of a 2.5% phenol red dye indicator, sterilized using a 0.45-micron filter. The medium was well-mixed and poured into sterile Petri dishes, which were stored at 4°C until use. The plates were inoculated with eight fresh yeast colonies using the streaking method and incubated at (28±2) °C for five days.

Estimation of L-Asparaginase (ASNase) Activity
The direct Nesslerization method (Cedar *et al*., 1968; Imada *et al*., 1973) was employed to estimate the activity of ASNase produced by the yeast strains ***P. kudriavzevii*** M64, ***H. uvarum*** M85, ***R. mucilaginosa*** M92, and ***M. guilliermondii*** M120, both mutated and non-mutated (used as control groups). The enzyme produced by the yeast catalyzes the hydrolysis of asparagine (used as a substrate) into aspartic acid and ammonia, according to the following equation:

$$\text{L-Asparagine} + H_2O \xrightarrow{\text{ASNase}} \text{Aspartic acid} + NH_3$$

The concentration of the resulting ammonia was estimated using spectrophotometric techniques. The absorbance intensity of the produced ammonia was measured at a wavelength of 450 nm for the complex formed by the reaction of ammonia with Nessler’s reagent, using a spectrophotometer.

Estimation of L-Asparaginase (ASNase) Activity in Culture Media
The samples produced L-Asparaginase (mutated and non-mutated by UV radiation) were activated and transferred to 200 mL of McDox broth. The cultures were incubated for 5 days at (28 ± 2)°C in a shaking incubator at 120 rpm to obtain the crude enzyme. The enzyme activity in the medium was then estimated using an assay with 0.5 mL of the culture medium, 0.5 mL of 0.08 M L-asparagine, and 1.0 mL of 0.05 M Tris-base buffer with a pH of 8.4. The mixture was incubated in a water bath at 37°C for 15 minutes. The reaction was stopped by adding 0.5 mL of 15% trichloroacetic acid (TCA). The precipitate was separated using centrifugation.

Enzyme activity was measured using the direct Nesslerization method after preparing the solution with EDTA and Nessler's reagent. The concentration of liberated ammonia was determined at a wavelength of 450 nm using a spectrophotometer. Enzyme activity was expressed in units of enzyme, defined as the amount of enzyme that releases one micromole of ammonia per minute under optimal conditions (Theantana *et al*., 2007). The activity was calculated according to the following equation:

$$\textbf{Enzyme Activity (Units/mL)} = \frac{\text{Concentration of liberated ammonia } (\mu\text{mol/mL})}{\text{Time of reaction (minutes)} * 14}$$

$$\textbf{Concentration of Liberated Ammonia} = \frac{\text{Absorbance at a wavelength of 450 nm}}{\text{Slope}}$$

The slope represents the relationship between absorbance intensity and ammonia concentration.

Estimation of Total Protein Concentration

The total protein content in the isolates *P. kudriavzevil M64*, *H. uvarum M85*, *R. mucilaginosa M92*, and *M. guilliermondii M120* was estimated according to the Bradford method (Bradford, 1976), which is based on the color change of Coomassie brilliant blue G-250 dye upon binding to proteins from brown to blue.

The intensity of the blue color was measured at a wavelength of 595 nanometers using a spectrophotometer, and the protein concentration was estimated using the following equation:

C = (A - b) / m

Where:

- **C**: Protein concentration (usually in micrograms/mL).
- **A**: Absorbance measured at a wavelength of 595 nanometers.
- **b**: y-intercept from the standard calibration curve.
- **m**: Slope of the standard calibration curve.

Purification of ASNase Enzyme Produced by the Yeast Strain *Meyerozyma guilliermondi* M120

The ASNase enzyme was purified as described earlier by Dioxin and Weeb (1961) by precipitation of enzymes and proteins by ammonium sulfate salts. The purification proceeded through ammonium sulfate precipitation, ultra and micro filtration, ion exchange and gel filtration.

The enzyme was firstly isolated from *Meyerozyma guilliermondi* M120 yeast through culturing the yeast in a suitable medium. The crude extract was spin at 6000 rpm for 15 minutes and the supernatant was collected for ammonium sulfate to make its concentration to 70% in ice with stirring for 30 minutes at 4°C and then let to stood at 4°C for 24 hours to allow the enzyme to precipitate. The precipitate was then sedimented at 6000 rpm for 6 hours and the deposit was suspended in sodium acetate buffer of a ration of 1:20 at pH 5.

Concentration of proteins was determined following Bradford’s method while the enzyme activity was determined before and after purification. Subsequently, the membrane filtration of the sample was carried out using a dialysis tube floated in sodium acetate solution under a magnetic stirrer at 4°C for 24 hours with an exchange of the buffer solution at 8-hour interval.

Ion-exchange chromatography was then performed with DEAE–Cellulose as a matrix; the matrix was activated with potassium phosphate buffer (pH 7). The enzyme solution was flows through the column via a 10 mL syringe and potassium phosphate probability solution was used in order elute the fractions with a rate of 30 mL/hour.

Finally, the gel filtration chromatography was made on Sephadex-G150 gel which was prepared in the same manner as that of the ion-exchange resin activation. The purified fractions were recovered using potassium phosphate solution at a flow rate of 30 mL/hour and enzyme activity and protein concentration were measured at each stage. The purified enzyme was then lyophilized to obtain it in powdered

form for further experiments, using a flask subjected to deep freezing at -80°C, followed by lyophilization at -60°C. (Whitaker and Bernard, 1972).

Characterization of L-asparaginase

The characteristics of the purified enzyme were determined, including the effect:

The buffer pH on enzyme activity

pH on the enzyme's stability

Temperature on enzyme activity and thermal stability.

Results and Discussion

Yeast Strains' Ability to Produce Asparaginase Enzyme

The results of testing the ability of eight non-mutated yeast isolates to produce the L-Asparaginase enzyme showed that none of the isolates were capable of producing the enzyme, except for the strain *M. guilliermondii* M120, which demonstrated a high production ability. A distinct pink halo was observed, indicating the enzymatic breakdown of the substrate (the amino acid L-Asparagine) by the asparaginase enzyme produced by the yeast, compared to control samples that lacked ASNase enzyme activity, as shown in **Figure (2).**

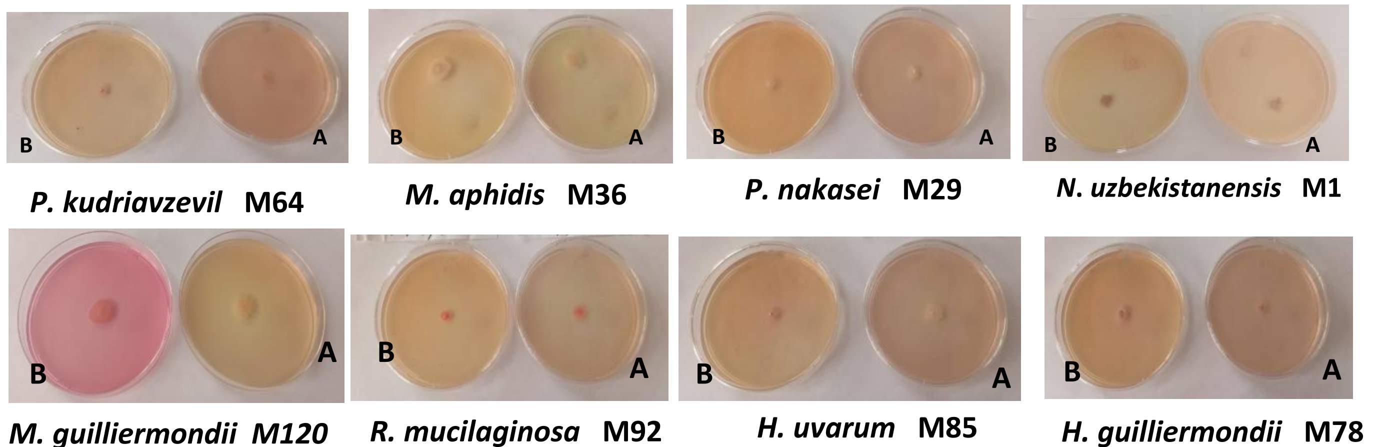

Figure 2: Yeast isolates producing and non-producing L-Asparaginase enzyme before mutation.
A: Control samples B: Treated samples

Upon exposing the yeasts to ultraviolet radiation, it was observed that the yeasts (*P. kudriavzevil* M64, *H. uvarum* M85, *R. mucilaginosa* M92) that were non-producers of the enzyme became producers, as evidenced by the formation of a pink halo around the yeast colonies. Additionally, the ability of *M. guilliermondii*

M120 to produce the enzyme increased compared to its production capacity before mutation, as shown in **Figure 3.**

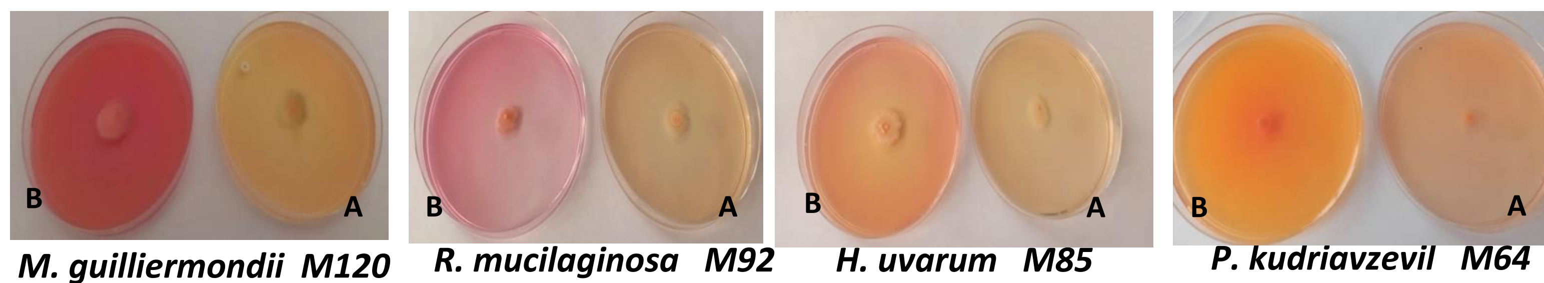

Figure 3: Yeast isolates producing L-Asparaginase enzyme after mutation.
A: Control samples **B: Treated samples**

Our results were consistent with those of studies conducted in this field. A study showed an increase in the production of L-Asparaginase enzyme from the wild strain of the marine fungus *Beauveria bassiana* SS18/14 when exposed to ultraviolet radiation, with a production of 8.34 units/mL compared to the non-mutated strain, which produced 6.32 units/mL (Kamala Kumari *et al*., 2015). Another study indicated that exposing bacterial cells to ultraviolet radiation resulted in a threefold increase in the production of L-Asparaginase compared to non-mutated strains (Prihanto *et al*., 2020).

L-Asparaginase Enzyme Activity and Total Protein Concentration

Measuring the L-Asparaginase enzyme activity using Nesslerization method showed the highest enzyme activity in the ultraviolet-mutated yeast strain *M. guilliermondii* M120, which reached 1.5 units/mL, compared to the non-mutated strain, which showed 1 unit/mL. Additionally, the protein concentration measured by the Bradford method showed an increase in protein concentration in the *M. guilliermondii* M120 strain from 0.32 to 0.45 mg/mL after UV mutation. In contrast, the *R. mucilaginosa* M92, *H. uvarum* M85, and *P. kudriavzevil* M64 strains had low enzyme activity and protein concentration before treatment, but showed significant improvement after treatment, as shown in **Table 4**.

Table 4: L-Asparaginase Enzyme Activity and Concentration in *M. guilliermondii* M120 Yeast Strain Before and After Ultraviolet Mutation

Enzyme-Producing Strain	Before Mutation	Before Mutation	After Mutation	After Mutation
	Enzyme Activity (Units/mL)	Protein Concentration (mg/mL)	Enzyme Activity (Units/mL)	Protein Concentration (mg/mL)
***M. guilliermondii* M120**	1	0.32	1.5	0.45
***R. mucilaginosa* M92**	0.07	0.14	0.24	0.5
***H. uvarum* M85**	0.04	0.9	0.19	0.4

P. kudriavzevil **M64**	0.03	0.06	0.16	0.3

Purification of L-Asparaginase from *Meyerozyma guilliermondii* M120 Yeast Isolate

Table (5) appear the results of L-Asparaginase purification from the *M. guilliermondii* M120 yeast isolate, including protein concentration and specific activity. It appears that there is a gradual decrease in protein concentration along with an increase in specific activity at each step of purification. In the crude extract, the protein concentration was 0.45 mg/mL, and the specific activity was 3.3 units/mg protein. After ammonium sulfate precipitation, the protein concentration decreased to 0.3 mg/mL, and the specific activity increased to 15 units/mg protein. After the final step of purification using gel filtration, the protein concentration further decreased to 0.1 mg/mL, while the specific activity reached 30 units/mg protein.

Table 5: Purification Results of L-Asparaginase Enzyme Produced by *M. guilliermondii* M120

Purification Step	Volume (mL)	Enzyme Activity (Units/mL)	Protein Concentration (mg/mL)	Specific Activity (Units/mg Protein)	Total Activity (Units)	Purification Fold	Recovery (%)
Crude Extract	75	1.5	0.45	3.3	112.5	1	100
Ammonium Sulfate Precipitation (70%)	20	4.5	0.3	15	90	4.5	80
DEAE-Cellulose Ion Exchange	21	3.5	0.15	23.3	73.5	7	65.3
Sephadex G150 Gel Filtration	18	3	0.1	30	54	9	48

Notes:

A: Enzyme activity = Reciprocal of the highest dilution × 1000 / Volume of L-Asparaginase added.

B: Total activity = Activity × Volume of L-Asparaginase.

C: Protein concentration (mg) = Bradford Method (1976).

D: Specific activity = Activity for that step / Total protein concentration for that step.

E: Purification fold = Specific activity for that step / Specific activity of the crude extract.

The table indicates a gradual decrease in protein concentration with an increase in specific activity at each step of purification. In the crude extract, the protein concentration was 0.45 mg/mL, and the specific activity was 3.3 units/mg of protein. After ammonium sulfate precipitation, the protein concentration dropped to 0.3 mg/mL, with a significant increase in specific activity to 15 units/mg protein. The final step of gel filtration resulted in a protein concentration of 0.1 mg/mL and a specific activity of 30 units/mg protein.

DEAE-Cellulose Ion Exchange Chromatography: The DEAE-Cellulose ion exchange method, based on charge differences for chromatographic separation, revealed a single protein peak in the wash step, concentrated in fractions (7-33) as shown in Figure 4. Another peak appeared during the elution step with a sodium chloride gradient (0-3 M), and enzyme activity in the eluted fractions showed an increase to 23.3 units/mg protein. The purification fold increased to 7 times, with a yield of 65.3%.

Gel Filtration Chromatography: The final purification step, gel filtration chromatography using Sephadex G-150, produced a homogeneous peak for L-Asparaginase, as seen in Figure 5. The enzyme activity was maximized at fraction 21, showing a specific activity of 30 units/mg protein. The total recovery was 48%, and the purification fold reached 9.

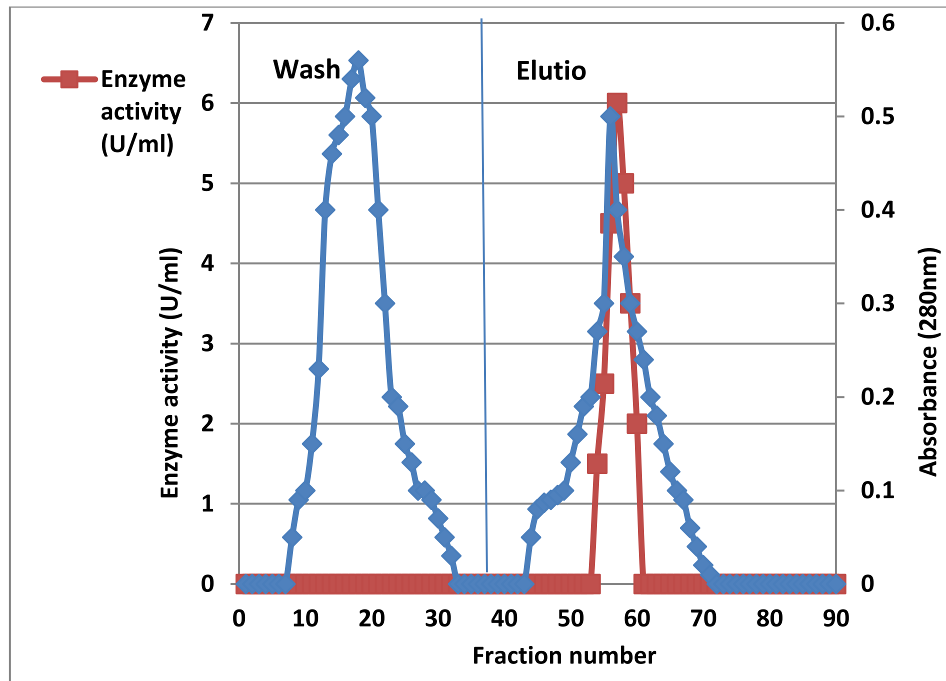

Figure (4): Purification of L-Asparaginase from *M. guilliermondii* M120 Yeast Using DEAE-Cellulose Ion Exchange in a 2.5 × 20 cm Column

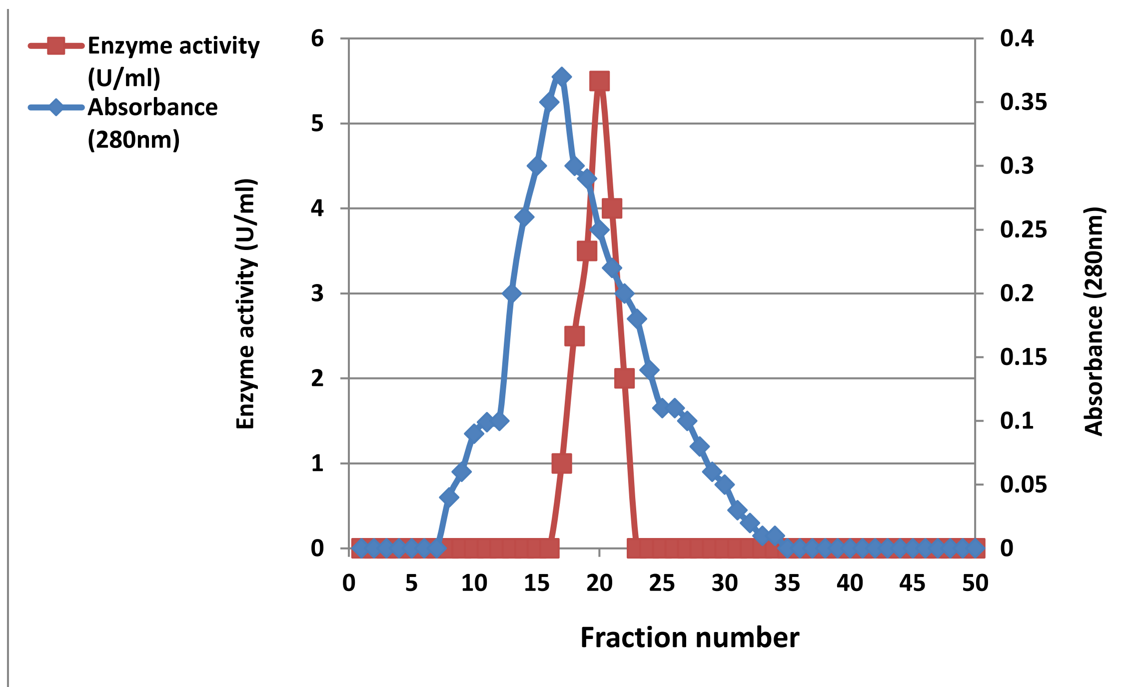

Figure (5): Purification of L-Asparaginase from *M. guilliermondii* M120 Yeast Using Gel Filtration Chromatography on Sephadex G-150 Column

It is important to note that the concentration of L-Asparaginase using ammonium sulfate is a widely used method because it has a fast solubility rate,

thus allowing the precipitation of proteins based on the concentration and type of salts used in the enzyme concentration step. The precipitation of proteins occurs due to the neutralization of their charges with those of ammonium sulfate, leading to the aggregation and separation of proteins in a specific phenomenon called "salting out." A study by da Silva Duarte *et al*. (2024) revealed that L-Asparaginase was purified from *Aspergillus niger* through a series of steps, starting with enzyme concentration using ammonium sulfate at an 80% saturation level. Specific activity detected for the enzyme in this step was 5.44 units/mg; the purification fold obtained was 3.32 and the enzyme recovery was 14.97%. The subsequent step with DEAE ion exchanger enhanced the specific activity to 12.33 units/mg, purification fold to 7.52 and 4.80% yield. The enzyme was successfully purified from *Leucosporidium scottii* L115 yeast by Sanchez-Moguel et al. (2023) using DEAE-Cellulose ion exchange chromatography with specific activity and purification fold of 14.65 units the enzyme was, however, obtained in the dry form as a powder. (Figure 6).

Figure (6): L-Asparagenase enzyme powder

Characterization of Purified L-Asparaginase Enzyme

1. Effect of pH on Asparaginase Activity

The results of testing the effect of pH on asparaginase activity within a pH range from 5 to 10 showed a gradual increase in enzyme activity from pH 5 to 7, followed by a gradual decrease in activity beyond this pH (Figure 7).

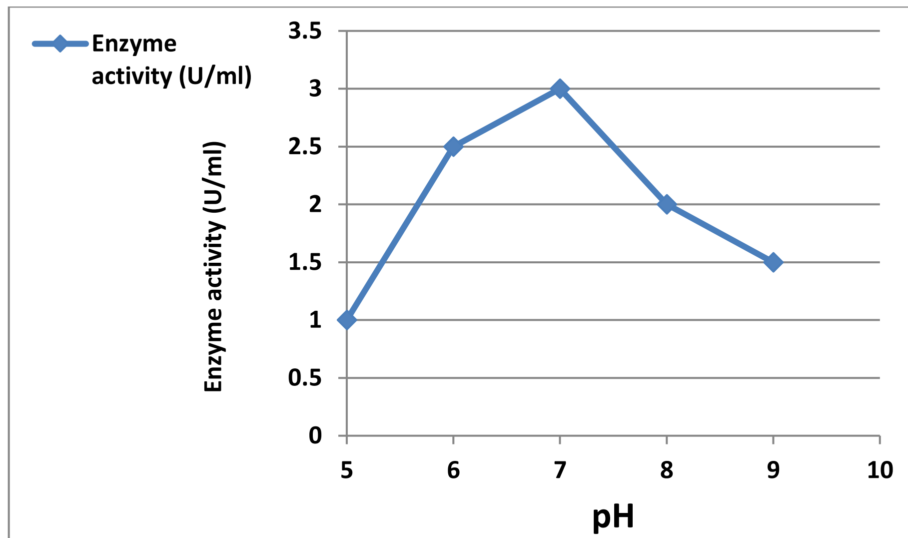

Figure (7): Effect of pH on the activity of purified L-Asparaginase enzyme produced by *M. guilliermondii* M120.

In comparison with previous studies, Benchamin *et al*., 2019 reported that pH 8 provided the optimal activity for L-Asparaginase enzyme extracted from *Aspergillus fumigatus*. It was also found that the optimal activity of L-Asparaginase produced from different fungal species, including *Aspergillus lentulus* and *Rhizopus spp*., occurred within the pH range of 5-7 (Damare and Kajawadekar, 2020; Ranjini Priya and Subhashini, 2022).

2. Stability at pH of L-Asparaginase

The results of thermal stability showed that L-Asparaginase enzyme was more stable at pH 7 and 8, where the enzyme retained the highest residual activity (100%) at these pH values. However, its residual activity decreased at pH levels lower or higher than these values (Figure 8).

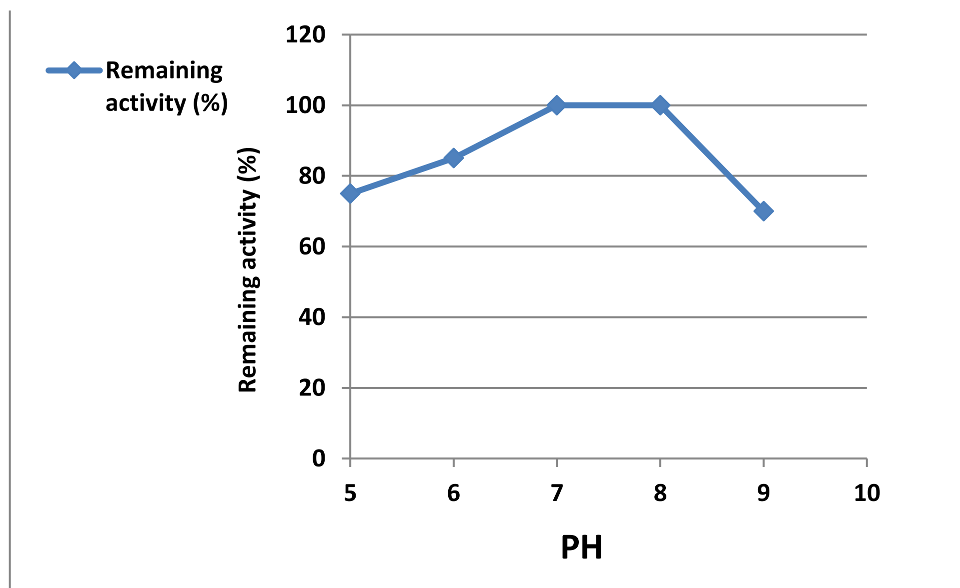

Figure (8) pH Effect on the Stability of Purified L-Asparaginase Enzyme Produced by M. Guilliermondii M120

Studies conducted in this field have indicated that L-Asparaginase enzyme extracted from the fungus *Ganoderma australe* exhibits higher stability in neutral to slightly alkaline environments, with the enzyme showing greater stability at pH levels of 7-8 (Chakraborty and Shivakumar, 2023). This stability makes it suitable for applications in cancer treatment due to its high activity and stability under these conditions. Another study demonstrated that L-Asparaginase purified from the fungus *Fusarium solani* showed optimal stability and activity at pH 7 and 8 (Osama *et al*., 2023).

3. The Effect of Temperature on L-Asparaginase Activity

The enzyme's activity was measured at temperatures ranging from 27°C to 47°C

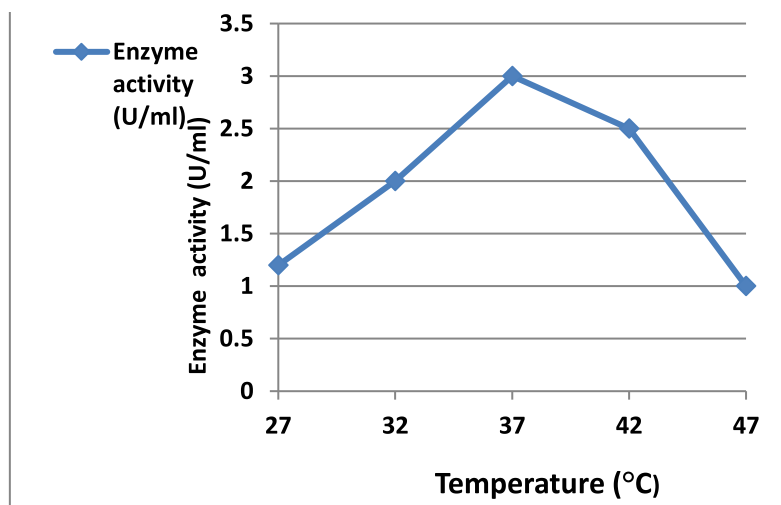

Figure (9): The Effect of Temperature on the Activity of Purified L-Asparaginase Produced by *M. guilliermondii* M120

The maximum enzyme activity was observed at 37°C, with a noticeable decrease in activity at temperatures lower or higher than this point (Figure 9). Our findings are consistent with other studies, which recorded similar results for L-asparaginase extracted from filamentous fungi at 37°C (Al Zubairy *et al*., 2023) and the fungus *Ganoderma australe* (Chakraborty and Shivakumar, 2023).

4.Thermal Stability of L-Asparaginase

Figure (10) illustrates that L-Asparaginase retained 100% of its activity when

incubated at temperatures between 32°C and 37°C. The enzyme began to lose its activity beyond this range, with the residual activity dropping to 55% at 52°C.

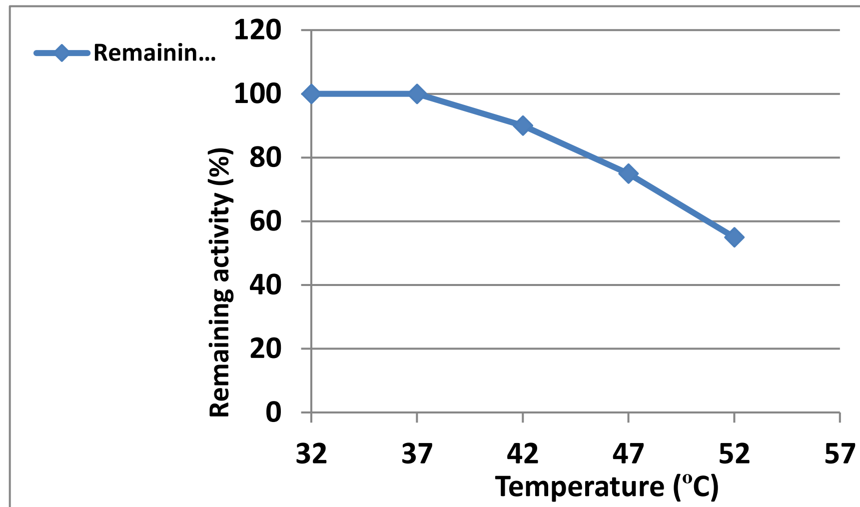

Figure (10): Thermal Stability of L-Asparaginase Produced by *M. guilliermondii* M120

In another study conducted by Lefin *et al*. (2023), it was observed that L-Asparaginase produced by fungi remained stable at temperatures ranging between 20°C and 37°C, but it significantly lost its activity at 60°C.

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