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Qualitative Screening and Activity Assay of L-asparaginase-Producing Fungi from Tempeh

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Abstrak: This study aims to isolate and determine the types of fungi in tempeh that are capable of producing the enzyme L-asparaginase and how the enzyme activity is produced. The following steps were taken: Fungi were isolated using the pour plate method with Potato Dextrose Agar (PDA) medium; morphological characterization of fungi was performed macroscopically and microscopically.The ability to produce asparaginase was assessed qualitatively using modified liquid M9 medium and quantitative assay of L-asparaginase activity with the Nessler reagent. Potential fungi were identified based on identification books. The results of fungal isolation from tempeh yielded of 7 fungal isolates. Qualitative selection of fungal isolates from tempeh capable of producing L-asparaginase resulted in 4 fungi capable of producing the L-asparaginase enzyme, indicated by a pink color change in the medium. Quantitative assay was performed using an enzyme activity test .The results of quantitative selection showed that the highest activity of L-asparaginase enzyme was found in fungal isolate with the code TP 3, reaching 16,386 U/mL, which has been identified macroscopically and microscopically and is classified as belonging to the genus *Aspergillus.* These findings indicate that fungi from tempeh have the potential to produce the enzyme L-asparaginase.
Keywords: Fungi; L-asparaginase; screening; tempeh

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INTRODUCTION

Tempeh is a traditional fermented soybean product that is widely consumed in Indonesia due to its rich flavor and high nutritional value. Tempeh is often used in both traditional and modern cuisine, making it an important component of the Indonesian diet. Per capita tempe consumption in Indonesia in2023 is 0.143 kg per capita per week, an increase of 2.31% from 2020 (Dewi et al., 2024) The fermentation of soybeans into tempeh involves microorganisms that degrade soybean proteins into smaller peptides, free amino acids, and various bioactive metabolites beneficial to human health. Among the amino acids released through this protein hydrolysis is asparagine (Prativi et al., 2023).

Asparagine is a non-essential amino acid that can be synthesized into aspartic acid. This compound is naturally found in plants such as potatoes, wheat, and various grains, including peanuts and soybeans. In the production of tempeh, which uses soybeans as the main ingredient, the fermentation process involves various microorganisms that are capable of converting asparagine into aspartic acid. This explains why the aspartic acid content in tempeh is quite high, reaching 124,62 mg / 100 g protein (Rahmi et al., 2018). Asparagine plays an important role in the formation of acrylamide. The higher the asparagine content in a plant, the greater the potential for acrylamide production. Acrylamide compounds appear when asparagine reacts with reducing sugars, such as glucose, under high heating conditions. Acrylamide is known to have negative effects on health, including neurotoxicity, carcinogenicity, and disruption of the reproductive system (Freitas et al., 2021). In many processed meals, the Maillard reaction which happens when reducing sugars and amino acids, particularly asparagine, are heated forms acrylamide, an undesirable and possibly cancer causing substance. Even while tempe fermentation takes place at relatively low temperatures in traditional food preparation, Maillard chemistry can be triggered and acrylamide precursors can be produced by subsequent thermal treatments like frying or roasting. This is especially important for tempe that contains a lot of asparagine since it could help acrylamide develop when heated. (Adimas et al., 2024)

The formation of acrylamide can be mitigated through the use of enzyme L-asparaginase, which hydrolyzes asparagine (Jia et al., 2021)**.** L-asparaginase (EC 3.5.1.1) is an enzyme that catal yzes the hydrolysis of asparagine into aspartic acid and ammonia. It plays a significant role in both healthcare and the food industry (Dinarvand et al., 2020). In medicine, L-asparaginase is widely used as a primary therapeutic agent in the treatment of acute lymphoblastic leukemia and lymphosarcoma, and its effectiveness as a chemotherapeutic enzyme has been well established (Ambreen et al., 2022). In the food industry, the enzyme is valued for its ability to reduce acrylamide formation. L-asparaginase catalyzes the hydrolysis of L-asparagine, thereby reducing the level of free asparagine in food ingredients. In the tempeh industry, the presence of natural L-aspa raginase from fermentation microbes provides an additional benefit, namely the potential to reduce the risk of acrylamide formation when tempeh is fried or baked. Moreover, L-asparaginase is commercially available from microbial sources (Adhikari & R., 2017)

Fungi in tempeh, such as *Rhizopus oryzae*, are the most commonly found and dominant types of mold and are the most important components in tempeh fermentation (Utari et al., 2010)**.** Recent research has proven that there is a new hyperactive L-asparaginase producer from the *Rhizopus* genus, namely *Rhizopus oryzae* AM16. This isolate is capable of producing enzymes in large quantities (2,875.9 U). The advantage of *R. oryzae* AM16 over bacteria (such as *E. coli*) is its eukaryotic nature, which researchers believe can reduce the potential for immunological reactions when used as a therapeutic agent. The anticancer activity of the purified L-asparaginase from *R. oryzae* AM16 against several cancer cell lines found that dialysis and purification processes increased its cytotoxic potential (Othman et al., 2022). Tempeh contains various types of fungi, including *Rhizopus* are considered promising natural sources of L-asparaginase. However,no studies have yet reported the potential of fungi isolate from tempeh as a source of L-asparaginase.

Therefore, to explore the potential of fungi from tempeh, this study isolated and screened fungi derived from tempeh to determine their ability to produce L-asparaginase. This study aims to isolate and determine the types of fungi in tempeh that are capable of producing the enzyme L-asparaginase and how the enzyme activity is produced.

METHOD

Isolation of Microorganisms from Tempeh

The microorganisms to be isolated consisted of fungi and bacteria. A total of 10 g of tempeh sampel was aseptically homogenized using a sterile mortar and suspended in 90 mL of sterile distilled water to obtain 10^{-1} dilution. Serial dilutions were then prepared up to 10^{-3}. An aliquot of 0.1 mL from the final dilution was inoculated onto Potato Dextrose Agar (PDA) for fungal isolation.The culture plates were incubat ed at room temperature, and colony growth was o bserved daily. Fungal colonies gromn on PDA were subcultured onto fresh PDA, subculturing was repeated several times until pure isolates were obtained.

Macroscopic and Microscopic Characterization

Pure isolates of fungi were then characterized macroscopically. Macroscopic characterization of fungi were characterized by colony color, colony texture and colony growth. Macroscopic character observation of fungi was performed using slide culture method with lactophenol blue cotton stain, then observing the characteristics and color of hyphae, the presence or absence of conidia, the shape and color of conidia.

Qualitative Screening of Fungi Producing L-asparaginase

Qualitative selection based on the ability of fungi and bacteria to produce acid in media containing asparagine (Al Zubairy et al., 2023). The medium used was M9 supplemented with 0,5 % phenol red as a pH indicator. The composition of M9 medium per liter was as follows: 6 g Na_2HPO_4. $2H_2O$, 3 g KH_2PO_4, 0,5 NaCl, 5 g L-asparagin, 0,5 g $MgSO_4.7H_2O$, 0,014 g $CaCl_2.2H_2O$, and 20 g glucpse (2% w/v). Each fungal and bacterial isolate was inoculated onto M9 medium and incubated at room temperature. colony growth was monitored daily. A change in the color of the medium from yellow to pink indicated L-asparaginase activity (Han et al., 2014). Isolates that showed a positive reaction were selected for further analysis.

Production of L-asparaginase

For L-asparaginase production, 100 mL of M9 medium supplemented with 1% (w/v) L-asparagine was dispensed into 500 mL Erlenmeyer flasks. Each flask was inoculated with 2 mL (2% v/v) of culture from the qualitatively selected isolate. The cultures were incubated at 27 °C on a rotary shaker at 10.000 rpm for 5 days. Following incubation, the culture broth was centrifuged at 10.000 rpm for 10 min, and the resulting clear supernatant was collected as the crude enzyme extract for subsequent assays (Badoei-Dalfard, 2016).

Quantitative assay of L-asparaginase Activity

0,1 mL of crude L-asparaginase enzyme extract was added to 0,4 mL of 0.2 M Tris-HCl buffer (pH 8.6) and 1 mL of 0.01 M L-asparagine and incubated for 30 minutes at 37 °C. The reaction was stopped by adding 1 ml of 1.5 M trchloroacetic acid (TCA), and the precipitated protein was removed by centrifugation at 3400 rpm for 15 minutes. Subsequently, 0.5 mL of resulting supernatant was mixed with 7 mL of distilled water and 1 mL of Nessler's reagent (Imada et al., 1973). The mixture was left to stand for 15 minutes, after which absorbance was measured at 450 nm using UV-Visible spectrophotometer. The amount of ammonia released during the enzymatic reaction was quantified against a standard curve prepared with ammonium sulfate. All assays were conducted in triplicate. Enzyme activity was expressed in International Units (IU), where one unit is defined as the amount of enzyme required to liberate 1 µmol of ammonia per mL per minute (µmol/mlmin) under the assay conditions. Asparaginase activity is calculated as follows: (Nafisaturrahmah et al., 2023).

$$\text{Aktivitas Enzim (IU/ml)} = \frac{y-b}{a} \times \frac{\mathrm{vt}}{\mathrm{va}} \times \frac{1}{\mathrm{ve}} \times \frac{1}{Ti}$$

Keterangan:

y : absorbance	Va : Total volume analyzed
a : slope	Ve : Enzyme volume analyzed
b : intercept	*Ti* : Enzyme incubation time
Vt : Total volume	

RESULT AND DISCUSSION

Isolation And Characterization of Fungi From Tempeh

The purpose of fungal isolation is to obtain fungal isolates found in tempeh products. The tempeh used for isolation was incubated for 3 days after the first day of production. From the isolation results using Potato Dextrose Agar media (PDA). PDA medium is one of the most commonly used culture media for growing and identifying fungi, as well as enabling observation of sporulation color and colony growth patterns that aid in the initial morphological identification process (Yuliana, et al., 2022). Macroscopic observation of fungal characteristics includes colony color, colony texture, and colony growth. Microscopic observation of fungi is performed using the Slide Culture method with Lactofenol Blue Cotton stain. then the characteristics and color of the hyphae, the presence or absence of conidia, and the shape and color of the conidia are observed.

Table 1. Macroscopic and microscopic characterzation of fungi from tempeh

Isolate	Macroscopic Morphology				Microscopic Morphology
	Colony shape	Margin	Color	Texture	
TP	Circular	Entire	Whitish-gray	Cottony	Hyaline hyphae, non-septate
TD	Circular	Entire	Whitish-gray	Cottony	Spherical sporangium, long sporangiophore, non-septate hyphae
TP 1	Circular	Entire	White	Cottony	Spherical vesicle with phialides, conidia forming chains
TP 3	Circular	Irregular	White with brown spots	Granular	Round conidia, septate hyphae, pigment production observed
TP 2	Circular	Entire	White	Cottony	Septate hyphae, upright conidiophore, round vesicle with terminal conidia
TP 4	Circular	Lobate	Whitish-gray with brown pigment	Granular	Branched conidiophores, dark conidia, septate hyphae
TD 1	Circular	Entire	White with Yellowish center	Cottony	Conidiophore with spherical vesicle, dark-colored conidia

Notes: TP (Tempeh in plastic package), TD (Tempeh in leaves package)

Based on macroscopic and microscopic observation, the following results was obatained 7 fungal isolates were successfully grown. Of all the fungal isolates, 3 of them were suspected to belong to the genus *Rhizopus* sp. This was based on macroscopic observations showing isolates with the codes TP, TP 1, and TD with grayish-white mycelium resembling *Rhizopus oligosporus* and *Rhizopus oryzae* fungal colonies (Pitt & Hocking, 2009). *Rhizopus* fungi, particularly *R. oligosporus*, are the main microorganisms responsible for tempe fermentation. This dominant fungus can grow rapidly on soybean substrate and form mycelium that binds soybeans together to form tempeh. *R. oligosporus* can also produce various enzymes, including protease and lipase, which function to hydrolyze soybean fats and proteins, thereby increasing digestibility and imparting a distinctive flavor. In addition to *R. oligosporus*, there is a secondary fungus that is also commonly found in tempeh, namely *R. oryzae*. (Sine & Soetarto, 2018). Meanwhile, isolates TP2, TP3, TP4, and TD1 exhibited conidiophore structures with round, brown or yellow pigmented vesicles and conidia chains that were more similar to the characteristics of *Aspergillus* sp. in fermented foods (Pitt & Hocking, 2009). *Aspergillus* sp. is a species of fungus that contaminates foodstuffs, including tempeh. In tempeh stored for more than 1 day at room temperature, non-harmful types of Aspergillus were found (Atika et al., 2019). In tempeh production, *Aspergillus oryzae* can be used as a fungal inoculum for tempeh fermentation (Hartanti et al., 2015). Studies that conducted mycotoxin testing on food stated that there are Aspergillus species that produce small amounts of mycotoxins. (Blumenthal et al., 2004).

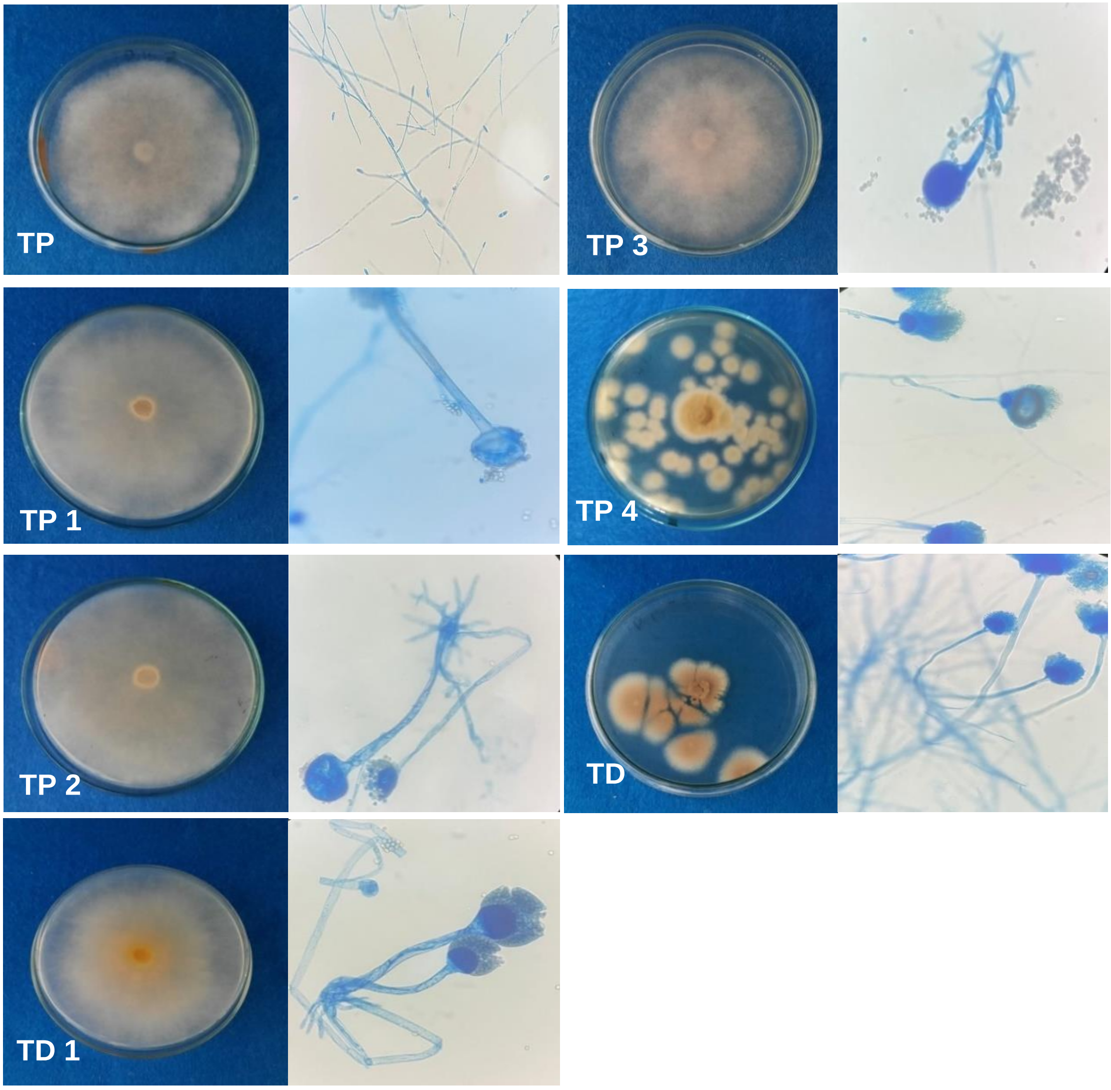

Figure 1. Macroscopic and microscopic characterization of fungal isolates from tempeh with lactophenol blue cotton stain.

Qualitative Screening of L-asparaginase-Producing Fungal Isolates From Tempeh

Qualitative screening of L-asparaginase-producing fungal isolates from tempeh showed that 4 isolates were capable of producing L-asparaginase, indicated by a change in the color of the solution from yellow to pink. The six were then clarified with quantitative tests through enzyme activity determination.

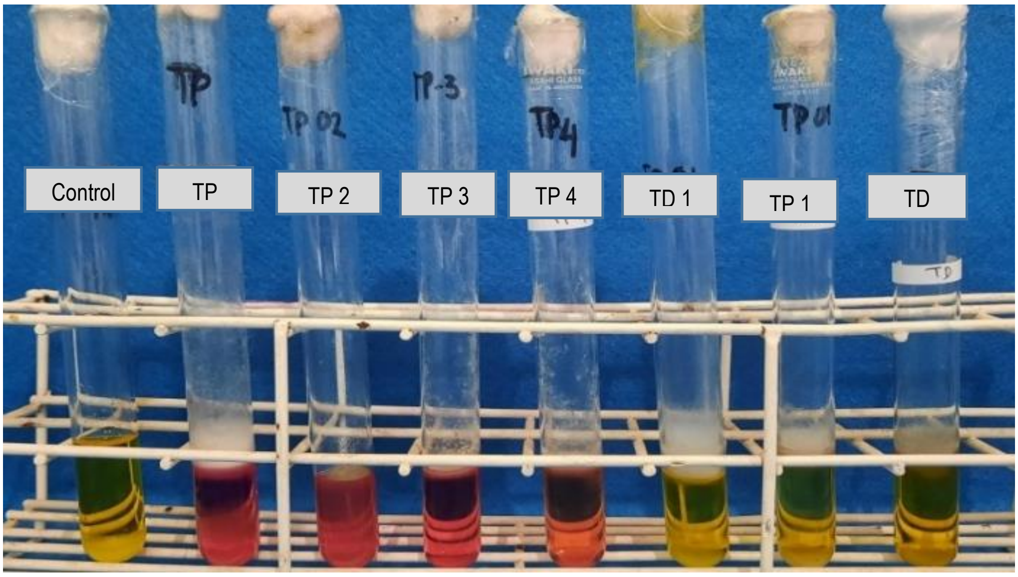

Figure 2. Screening of L-asparaginase activity producing fungal isolates from tempeh after 5 days of incubation. Control: M-9 medium without fungal isolates

The isolates were inoculated on modified M9 liquid medium containing 1% L-asparagine and phenol red as indicator. Based on the screening results of 7 fungal isolates isolated from tempeh, 4 isolates showed positive results marked by a change in the color of the medium to pink, and 3 isolates showed ne gative results with no change in the color of the medium, meaning that these isolates could not produce L-asparaginase this suggests that L-asparagine is not hydrolyzed by the isolate, therefore no ammonia is released into the medium and yhe medium shows no change in pH or no color cahnge in the indicator observed.L-asparaginase producing isolates hydrolyzed L-asparagine to aspartic acid and ammonia (NH_3), increasing the pH (basa) of the medium indicated by conversion of phenol red color to pink (Osama et al., 2023) 4 fungal isolates showed a greater intensity of pink coloration (Figure 2).

L-asparaginase Quantitative Assay

The determination of crude L-asparaginase enzyme activity produced by fungi isolated from tempeh was carried out using the Nessler method. Ammonia released by L-asparaginase enzyme hydrolysis reacts with Nessler. The Nessler reagent is sensitive to ammonium in alkaline solutions and will form a brownish-yellow dispersed colloidal complex. The color intensity in the sample is then measured spectrophotometrically. The absorbance value is then regressed with a standard ammonium chloride curve (Juliasih et al., 2024). One unit of L-asparaginase (U) is defined as the amount of L-asparaginase enzyme that catalyzes the formation of one µmol of ammonia per minute under test conditions (Basha et al., 2009). The following are the results of the crude L-asparaginase enzyme activity test:

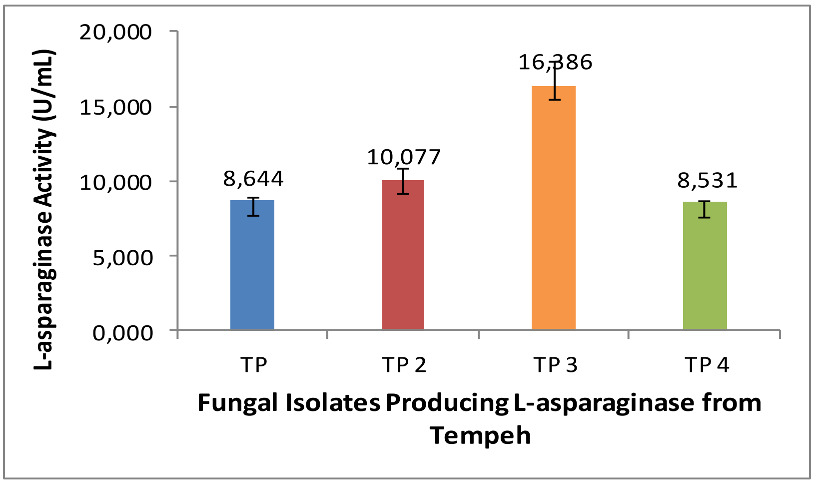

Figure 3. Diagram of the L-asparaginase Activity Assay of Fungal Isolates From Tempeh Using the Nessler Assay.

Based on the results of this study, it is known that the L-asparaginase enzyme activity of fungi isolated from tempeh shows varying results. Asparaginase activity can be seen from the high ammonia levels found in each sample. The higher the ammonia levels in the sample, the higher the asparaginase activity in the sample.The lowest L-asparaginase activity was found in isolate TP4 (8,531 U/mL), while the highest L-asparaginase activity was found in fungal isolate TP3 (16,386 U/mL) (Figure 3.). Differences in L-asparaginase enzyme activity among fungal isolates isolated from tempeh are influenced by genetic differences among the fungal isolates. Differences at the gene and gene expression levels result in large variations in the activity of the enzymes produced. In addition, researchers concluded that enzyme production is regulated by the nitrogen source in the medium (Cachumba et al., 2016).

In this study, isolate TP3 has been identified microscopically and macroscopically, suggesting that this fungus belongs to the *Aspergillus* sp. This fungus is an important component and is often found in the fermentation process of traditional foods, including tempeh. The specific role of this fungus in fermentation varies depending on the substrate used. This fungus plays an important role in breaking down complex compounds into simpler forms, increasing the digestibility and nutritional availability of fermented foods (Oktavia et al., 2024). In previous studies on microorganisms capable of producing the enzyme L-asparaginase, the genus *Aspergillus* sp. was also included among fungi with the potential to produce L-asparaginase, including the isolate *Aspergillus candidus* UCCM00117 with a specific activity of 23,739 IU/mg before purification (Ekpeyong et al., 2021). Other studies also reported that the fungus *Aspergillus aculateus* is also capable of producing L-asparaginase enzyme reaching 207 IU/mg (Dange et al., 2011).

CONCLUSION

Based on the results of the study, it can be concluded that there are 7 isolates that were successfully isolated from tempeh products packaged in plastic and leaf wrappings. Four isolates from tempe have the potential to produce L-asparaginase enzyme after qualitative screening and enzyme activity testing, with L-asparaginase activity values ranging from 8.531 U/mL to 16.386 U/mL. The highest activity value was found in the fungal isolate with the code TP3, which was suspected to be from the results of macroscopic and microscopic identification as a fungus from the genus *Aspergillus* sp.

RECOMMENDATION

Based on research findings demonstrating that fungi originating from tempeh have the potential to produce L-asparaginase, further exploration and investigation are required. These should include protein content analysis to dtermine the specific activity of the L-asparaginase produced, as well as optimization and purification of the enzyme. Such steps are necessary to obtain accurate activity data and improve the quality and applicability of the L-asparaginase generated.

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