Iranian Journal of Microbiology
Volume 18 Number 3 (June 2026) 439-447
DOI: http://doi.org/10.18502/ijm.v18i3.21671

ORIGINAL ARTICLE

Cellulase and β-glucosidase activity of crude enzyme extracted from fresh and expired soybean tempeh

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Received: July 2025**, Accepted:** May 2026

ABSTRACT

Background and Objectives: Enzymes are protein biomolecules that act as catalysts, including cellulase and β-glucosidase with extensive applications. Thus, this work aimed to contrast the activity of both enzymes from tempeh fermented by *Rhizopus microsporus* at 2 and 7 days of incubation.
Materials and Methods: Incubated tempeh was tested for quality evaluation. The crude extracts of cellulase and β-glucosidase were obtained by extracting tempeh with a cold phosphate buffer solution. The presence of the *R. microsporus* was examined through microscopic identification, and molecular identification using PCR amplification. Cellulase activity was determined using 3.5-dinitrosalicylic acid (DNS) reagent, whereas β-glucosidase activity was evaluated by measuring the release of p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside (p-NPG).
Results: The protein content and water content increase with the length of fermentation time. Microscopic identification and molecular identification confirmed the presence of *R. microsporus*. The highest cellulase activity was found in fresh tempeh (2-day incubation) at 0.0911 U/mL at pH 7, while the highest β-glucosidase activity was found in expired tempeh (7-day incubation) at 0.0042 U/mL at pH 5.
Conclusion: These findings indicate that the standard quality contributed to the differences in the enzymatic profile of tempeh incubated for 2 and 7 days.

Keywords: Cellulase; β-glucosidase; *Rhizopus microsporus*; Tempeh

INTRODUCTION

Cellulase is one of the enzymes that play an important role. Cellulase is an extracellular enzyme that hydrolyzes the β-1,4 glycosidic linkages to convert cellulose into glucose. Cellulase is classified as three components namely exo-β-1,4-glucanase, endo-β-1,4-glucanase, and β-1,4-glucosidase. These components aid in the process of converting cellulose into glucose (1). Cellulase is often produced through fermentation processes by bacteria and fungi such as *Aspergillus niger*, *Aspergillus nidulans*,

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Aspergillus oryzae, *Rhizopus oryzae*, and *Rhizopus oligosporus* (2).

Cellulase has a very wide potential that can be used in an industrial scale. Cellulase is used in the textile industry for denim bio-stoning and bio-polishing (3). In the food industry, cellulase is applied in fruit ripening, enhancing the ability to filter vanilla extract, extracting flavoring agent, and essential oils (4). In the pharmaceutical industry, cellulase is used as an excipient in controlled drug release systems (5).

β-glucosidase is a key enzyme in glycoside hydrolysis. This enzyme primarily functions to enhance aroma and flavor during the fermentation process, such as in wine production, where it hydrolyzes glycosides into simpler, more aromatic compounds (6). β-glucosidase finds additional application in the production of ready-to-drink black tea. This enzyme can release volatile compounds that enhance the aroma and quality of the tea. It has been reported that β-glucosidase is utilized in speed up manufacturing processes without compromising quality (7). This enzyme functions as an indicator related to organic subtances decomposition in soil. β-glucosidase activity is potentially affected by environmental conditions, for example moisture content and pH, and can be used to monitor the decomposition process in various types of land use (8).

Cellulase and β-glucosidase also contribute to the application of tempeh fermentation (9-11). *R. oryzae* and *R. oligosporus* can produce cellulase and β-glucosidase (12). Tempeh is a functional food produced from soybeans through fermentation by *Rhizopus* sp, covered by white mycelium, resulting in a compact and dense texture (13). According to the Food and Agriculture Organization (FAO) 2015, tempeh contains about 15% protein. Tempeh is generally consumed in its fresh form as a fermented food. However, in some regions, especially in Central Java, there is a cultural phenomenon where expired tempeh is used as a seasoning to achieve a distinctive aroma, a slightly bitter taste, and a unique sensation (14). Transformations in aroma, taste, and texture in this tempeh are suspected to be related to cellulase and β-glucosidase activity. However, scientific studies on the activity related to cellulase and β-glucosidase in fresh and expired tempeh remains restricted. Consequently, this study aims to investigate the profiles of fresh tempeh (2-day incubation) and expired tempeh (7-day incubation), as well as the molecular identification of one of the dominant *Rhizopus* spp.

MATERIALS AND METHODS

Tempeh production. Tempe was made from soybeans and fermented using yeast from local industries. About 2 kg of soybeans were boiled for 30 minutes, drained, and then soaked in distilled water for 24 hours. After peeling, the soybeans were boiled again until fully cooked, then drained and cooled before being evenly mixed with 15 g of yeast. The mixture was further packed in an airtight plastic container with proper air circulation and left to ferment for 7 days. Water and protein content were evaluated on days 2 and 7 following SNI 3144:2009 standards (15, 16).

Microscopic identification of fungi in fresh tempeh. Microscopic identification of fungi (Yazumi 107BN, ReHaze, and Sinher., China) was carried out using a chloral hydrate and aquadest reagent with 100x magnification to clarify the fungal structure, including sporangium, hyphae, and characteristic fragments of certain fungal species.

Enzyme extractions. About 7.5g of tempeh from 2 and 7 days of fermentation was weighed (AP125WD, Shimadzu®., Japan) and added to 30 mL of cold phosphate buffer, blended for 2 minutes (HR2221/30 350W, Royal Philips®., Netherlands), and centrifuged at 4000 rpm for 20 minutes (U-32R, Boece®., Germany). The supernatant was subsequently used for enzyme activity test (17).

Screening, isolation and identification of tempeh yeast. Yeast was obtained from local industries, screened, isolated, and identified for dominant fungi. Screening and isolation were performed using repeated inoculation on sabouraud dextrose agar (SDA) medium and incubated at 25°C for 5 days. The fungi were visually observed for color uniformity to ensure purity. The first incubation showed color diversity, hence, a re-inoculation was performed. In this study, the inoculation was repeated up to the sixth generation, resulting in a homogeneous white color.

Molecular identification. Fungi identification was performed molecularly by extracting genomic DNA using the Quick-DNA Magbead Plus Kit according to the manufacturer's instructions. The DNA target was amplified twice with PCR using MyTaq HS Red Mix. The ITS gene amplification product was analyzed

by electrophoresis, then bidirectionally sequenced using the Sanger method with primers ITS1 (primer forward) 5' – TCCGTAGGTGAACCTGCGG – 3' and ITS4 (primer reverse) 5' – TCCTCCGCTTATTGATATGC – 3' with Capillary Electrophoresis (1st BASE subcontracted lab study). Subsequently, the results of Sanger Sequencing were analyzed bioinformatically, resulting in the Top 10 Hit BLAST against the NCBI database, which showed 100% query coverage for each species. However, the percent identity values varied among species, including *R. microsporus* isolate PU112 (MT279277.1), *R. microsporus* strain JJ-A3 18S (HQ285720.1), and *R. azygosporus* UICC 531 (LC514333.1) had the highest percent identity, at 99.71%. This was followed by *R. azygosporus* isolate PU14096 (MT259026.1), *R. microsporus* isolate PU483 (MT279281.1), *R. microsporus* strain 1M5 (MT620751.1), *R. microsporus* isolate PU141 (MT279278.1), *R. microsporus* strain SMM4 (MK396495.1), and *R. microsporus* strain 379N (OP103934.1) with 99.57%, and finally *R. microsporus* isolate PUJW004 with 92.29% (MT279282.1).

Effect of pH on cellulase enzyme activity. Tempeh that was fermented for 2 and 7 days was extracted using a cold phosphate buffer solution at various pH levels, namely 2, 3, 4, 5, 6, 7 and 8. One mL of each supernatant was added with 1 mL of 1% CMC solution, then vortexed, and incubated for 60 minutes at 37ºC (Unimax 1010, Heidolph®., Germany). Subsequently, 2 mL of 3.5-dinitrosalicylic acid (DNS) reagent was added and then heated at 95ºC in a water bath for 10 minutes, and cooled at room temperature. Measurement was carried out using UV-visible spectrophotometry (UV-1800, Shimadzu®., Japan) at 405 nm wavelength.

Cellulase enzyme data analysis. The activity was measured by converting the absorbance values obtained from the standard glucose concentration, calculated using the formula (18). Statistical analysis was conducted using Two-Way ANOVA, followed by Šídák's post-hoc test (GraphPad Prism 10, GraphPad Software, LLC., USA).

$$AE=\frac{C}{MW\ Product}\ x\frac{H}{E}$$

Abbreviations: AE = enzyme activity (U/mL); H = total of enzyme - substrate volume (mL); C = glucose concentration; MW = molecular weight of glucose (180g/mol); E = enzyme volume.

Effect of pH on β-glucosidase enzyme activity. The phosphate buffer solution, used for extracting tempeh fermented for 2 and 7 days, was adjusted to various pH levels, namely 2, 3, 4, 5, 6, 7, 8 and 9. Each supernatant was pipetted in a volume of 2.7 mL. Added 300 µL of 1 mM p-nitrophenyl-β-D-glucopyranoside (p-NPG) solution, vortexed, incubated at 50ºC for 30 minutes (Unimax 1010, Heidolph®., Germany), and then 300 µL of Na_2CO_3 reagent was added. Measurement was carried out using UV-visible spectrophotometry (UV-1800, Shimadzu®., Japan) at 405 nm wavelength.

β-Glucosidase enzyme data analysis. The activity was measured by converting absorbance value obtained from the concentration of the standard p-nitrophenol solution, calculated using this formula below, and statistical analysis was conducted using Two-Way ANOVA, followed by Šídák's post-hoc test (GraphPad Prism 10, GraphPad Software, LLC., USA).

$$\text{Enzyme activity}=\frac{(C \times 10 \times Fp)}{T \times MW\ p\text{-nitrophenol}}$$

Abbreviations: C = concentration of p-nitrophenol; T = incubation time (minutes); Fp = dilution factor; MW molecular weight of p-nitrophenol (139.11g/mol).

RESULTS

Tempeh production and extraction. Tempeh was fermented for 7 days. Then, quality evaluation was conducted on days 2 and 7. The evaluation of tempeh quality standards included water content at 105 ± 2°C, while protein content was measured using the Lowry method. Macroscopic observations revealed physical changes in tempeh incubated for 2 days compared to 7 days (Fig. 1), where there was a significant increase in protein content from 17.29% (2-day incubation) to 45.69% (7-day incubation). The increase in protein content was associated with an increase in tempeh moisture content in tempeh after 7 days of incubation, exceeding the standard limit (Table 1).

Fig. 1. Tempeh incubation: (A) 2 days. (B) 7 days

Table 1. Water content and protein content of tempeh in incubation 2 days (fresh), 7 days (expired).

Criteria	Tempeh (days)		References
	2	7	(SNI 3144-3:2015)
Water content	64.68%	67%	<65%
Protein content	17.29%	45.69%	>16%

Microscopic Identification of fungi in tempeh. In this study, microscopic identification of fungi was performed on fresh tempeh. The identification results, whether using chloral hydrate reagent or aquades reagent at 100x magnification, revealed aseptate hyphae and sporangium with sporangiospores that were globose to subglobose, some were smooth, large and irregular. The identification results are attached in Figs. 2A and B.

Screening, isolation and identification of tempeh yeast. The supernatant was obtained from tempeh extraction and incubated for 2 and 7 days to identify the fungi present in the starter culture. First, yeast was rejuvenated in lactose broth medium. After 5 days of incubation, fungal growth was observed, with colonies that were greyish-white, cotton-like in texture, and smooth in surface appearance (Fig. 3). Second, yeast purification was performed on Sabouraud dextrose agar (SDA) medium in Petri dishes and incubated for 3 days. The rejuvenated yeast was inoculated onto SDA medium and incubated for 3 days, resulting in first-generation purification, with a total of six generations obtained. The purification results showed fungal morphology similar to that observed during the rejuvenation process, with greyish-white colonies that had a cotton-like texture and a smooth surface.

Molecular identification. The isolated DNA was amplified by polymerase chain reaction (PCR) technique. Each amplification of all ITS sequences shows a DNA fragment with a single band at 700 bp (Fig. 4). The ITS sequence of fungi in tempeh (G-3233-1) was a similarity of 99.71% with *R. microsporus* isolate PU112 (MT279277.1), *R. microsporus strain* JJ-A3 18S (HQ285720.1) based on the results of the BLAST analysis (Gen-Bank), confirming the molecular identification of the isolate.

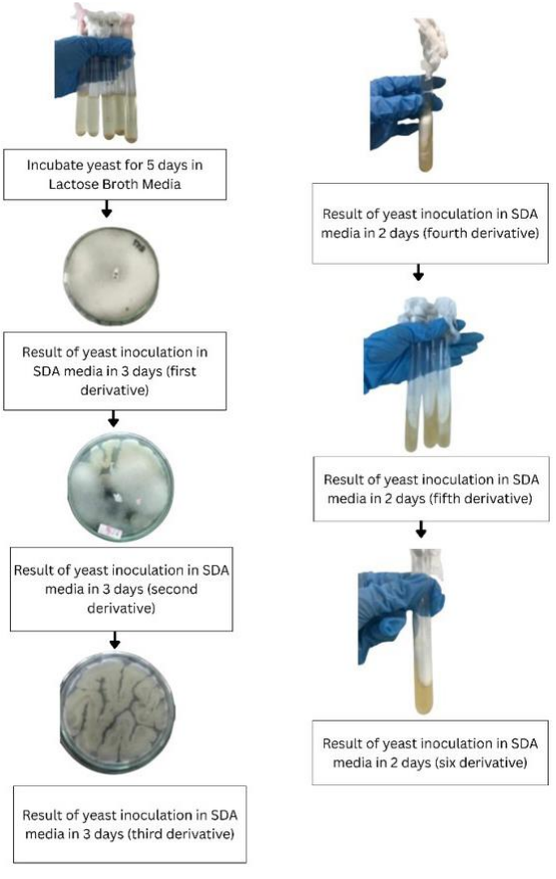

Fig. 3. Screening and isolation of tempeh yeast purification for 5 days.

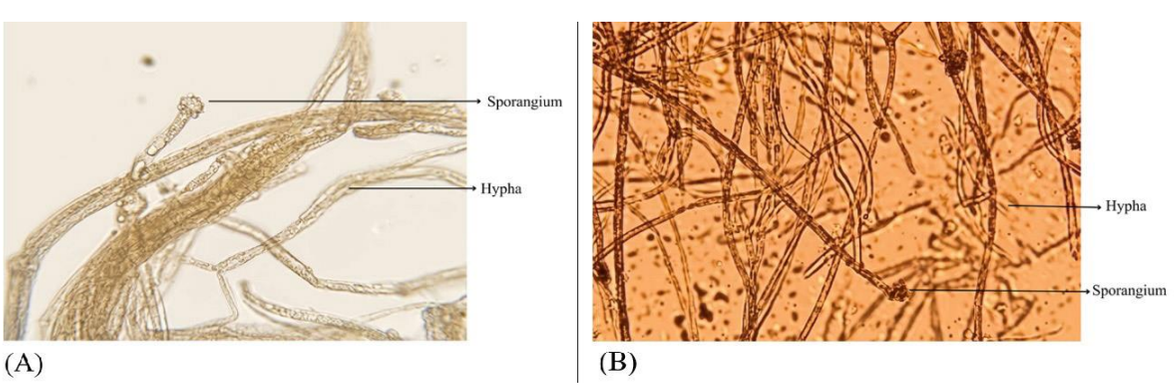

Fig. 2. Microscopic identification of fungi in tempeh. (A) Chloral hydrate reagent. (B) Aquadest reagen.

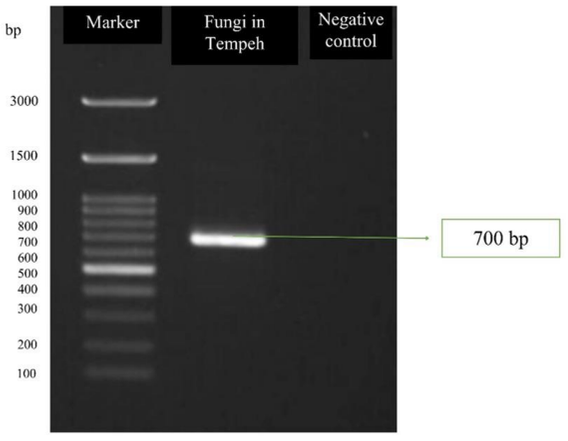

Fig. 4. Result of PCR amplification sequences of internal transcribed spacer (ITS) as representative.

Effect of pH on cellulase enzyme activity. The results of the Two-Way ANOVA analysis showed that pH significantly affected cellulase activity in tempeh incubated for 2 and 7 days ($p<0.0001$). Cellulase enzyme activity showed the highest results at pH 7 from tempeh incubated for 2 days. Based on the results, cellulase enzyme activity of tempeh incubated for 2 days and 7 days was 0.0911 U/mL and 0.0451 U/mL, respectively (Fig. 5). Further Šídák post hoc analysis showed that pH levels significantly affected cellulase activity ($p<0.0001$), except for pH 8, which showed a significant difference with $p=0.0024$ ($p<0.01$).

Effect of pH on β-glucosidase enzyme activity. The results of the Two-Way ANOVA analysis showed that pH significantly affected β-glucosidase activity in tempeh incubated for 2 and 7 days ($p<0.0001$). β-glucosidase enzyme activity showed the highest value at pH 5 in tempeh incubated for 7 days (0.0042 U/mL) and at pH 7 in tempeh incubated for 2 days (0.0034 U/mL) (Fig. 6). Further Šídák post hoc analysis showed that pH levels significantly affected β-glucosidase activity ($p<0.0001$), except for pH 8, which showed a significant difference with $p=0.0032$ ($p<0.01$).

DISCUSSION

Tempeh was fermented for 7 days (Fig. 1). Quality evaluation was conducted on days 2 and 7, based on the typical 2-3 day fermentation period needed for optimal results (19). The selection of 7 days is based on the optimal storage time for suitability for consumption. In the evaluation of standard tempeh

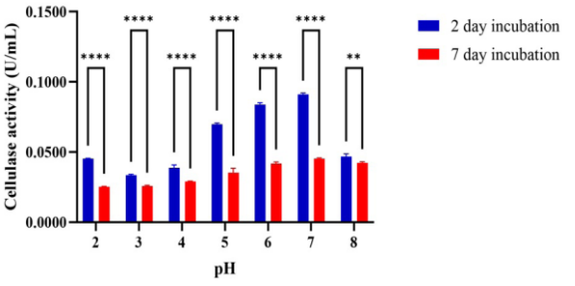

Fig. 5. Effect of pH on cellulase activity.

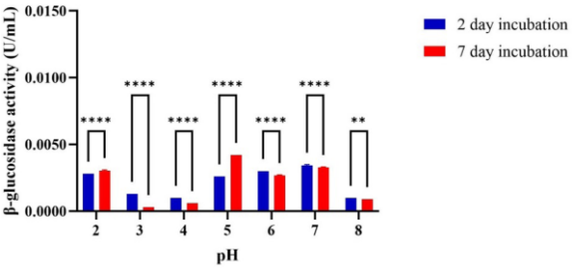

Fig. 6. Effect of pH on β-glucosidase activity.

quality, a significant increase in protein content was found from 17.29% (2-day incubation) to 45.69% (7-day incubation). The increase in protein content is suspected to be caused by the fermentation process (20). The fermentation duration influences the physical and chemical properties of tempeh. Physically, fermentation affects mycelial growth as well as the colour and aroma of tempeh. Meanwhile, chemically, it affects protein digestion, the degree of hydrolysis, and protein content, where these increases are caused by the activity of microorganisms from enzymes that degrade carbohydrate and protein substrates (21). Protein is a macromolecule composed of long chains of amino acids (22, 23). When the fermentation process is extended beyond 72 hours, amino acids increase and accumulate by the end of the fermentation process (24). This is suspected to be caused by proteolytic enzymes that hydrolyze long-chain protein molecules into shorter fragments (25). In addition, during the fermentation process, sugar components such as melibiose, raffinose, and sucrose gradually decrease over time (24). This contributes to the loss of dry matter carbohydrates as a result of hydrolysis and microbial metabolism (26), causing the protein proportion to increase as a result of relative changes. This correlates with the increase in water content observed from 64.68% (2 days of incubation) to

67.00% (7 days of incubation). The changes in the chemical components of tempeh after 2 and 7 days of incubation, including the increase in protein content, followed by the increase in water content, indicate a significant difference in the tempeh profile between 2 and 7 days of incubation. This difference in profile contributes to the enzyme activity profile produced, where the difference in chemical composition is known to correlate with microbiome activity over the course of fermentation (27).

Macroscopic identification showed physical changes between days 2 and 7 of fermentation. On day 2 of incubation, the surface of the tempeh is observed to be white, indicating the presence of gray-white fungal mycelium connecting the soybeans, forming a solid mass. Meanwhile, on 7 day of incubation, the texture of the tempeh visually appears watery, dark brown with spots that are observed to be brown and sometimes greenish (Fig. 1). This is related to the water content in tempeh. At 2 days of incubation, the water content was within an acceptable range, but at 7 days of incubation, it exceeded the maximum limit (Table 1), making the tempeh more susceptible to spoilage by microorganisms, including bacteria.

Microscopic identification of fungi in tempe revealed aseptate hyphae, single sporangiophore with sporangium that were globose to subglobose, some smooth, large and irregular, both in samples treated with chloral hydrate or aquades reagent (Figs. 2A and B). This finding was similar to the characteristic fragments found in *R. microsporus*, particularly the presence of aseptate hyphae and single sporangiophores with sporangiospores of various shapes (globose to subglobose) (28). In another study, unique characteristics were found in various strains of *Rhizopus* isolated from tempeh, including sporangiophores with more than two branches or a single sporangiophore bearing double sporangia. These characteristics were consistent with the morphology observed in *R. arrhizus*, *R. microspores*, *R. delemar* (29). This finding serves as supporting data to aid in molecular identification.

Screening, isolation, and identification of tempe yeast were carried out on the supernatant from tempe that had been incubated for 2 and 7 days. The first stage was carried out with yeast rejuvenation. Yeast rejuvenation is the process of providing optimal conditions so that yeast can grow and reproduce well. The yeast rejuvenation process is marked by media preparation, yeast addition, and the initial fermentation process to ensure that the yeast starts working and delivers the expected results. The media used in the yeast rejuvenation process for mold species is lactose broth. Lactose broth is a liquid medium that does not contain agar and is used for the growth of bacteria and fungi. Lactose broth provides the necessary nutrients for microorganisms, including carbohydrates (lactose) that can be used as an energy source. In the rejuvenation of yeast in tempeh, Then, the results of the yeast rejuvenation were inoculated onto sabouraud dextrose agar (SDA) medium and incubated for 3 days. The results of yeast rejuvenation and purification showed consistent outcomes in the form of fungal morphology with greyish-white colonies that have a cotton-like texture and a smooth surface. The selected strain was identified molecularly as *R. microsporus.*

Molecular identification through PCR amplification of the internal transcribed spacer (ITS) sequence showed a DNA fragment with a single band at 700 bp (Fig. 4), indicating successful and specific amplification of the target area. Molecular identification results showed that G-3233-1 had more than 99% similarity with *R. microsporus.* The fungi isolated from tempeh yeast is not a new species. Microorganism samples analyzed using the 16S rRNA marker are considered similar at the species or genus level when the "percentage identity" exceeds 97.5% or 95% respectively (30).

pH is an important parameter that can have an effect on enzyme activity (31). Cellulase enzyme activity showed the highest activity at pH 7 from tempeh incubated for 2 days (Fig. 5), indicating that the fungus in tempeh works optimally at neutral pH. Similar results were also reported in studies the synthesis of carboxy methyl cellulose (CMC) at pH 4-7 with *R. oryzae* under solid-state fermentation (SSF) produced the highest cellulase activity (32). Other studies support these results with the highest activity of cellulase enzyme activity at pH 7 in the genus *Geobacillus* (33, 34) and in *Streptomyces griseorubens* (35). However, different test results were found on cellulase enzyme activity from rice husk and rice bran fermented by *Trichoderma reesei* and *R. oryzae* reported optimal results at pH 6 (36). Differences in the best pH values show that cellulase enzyme can be actively produced in acidic to neutral pH environments (37). This is due to the wide growth of fungi, which occurs at pH 3 to 8 (38). It seems that the cellulase activity is higher in fresh compared to expired

tempeh. This also indicate the possible intention of using expired tempeh in cooking vegetable in Javanese cuisines is the flavor that might due to the degradation of protein in tempeh.

Regarding the effect of pH on β-glucosidase enzyme activity was quantitatively conducted on tempeh extract using 1M Na_2CO_3 and 1 mM p-NPG substrate. The β-glucosidase breaks down p-NPG into p-nitrophenol (yellow in color) and glucose, with activity measured through absorbance at 405 nm (39). This enzyme is very sensitive to pH, which affects activity. The highest activity was found at pH 5 for tempeh incubated for 7 days (Fig. 6). This difference can be attributed to the higher initial pH of fresh tempeh (6.5-6.9), resulting in lower enzyme activity during the 2 days incubation because the less acidic environment is not optimal for enzyme-producing microbes such as *Rhizopus* spp. (40). On the other hand, tempeh that has been incubated for 7 days has a lower pH as the incubation time increases. Other studies showed that the highest pH varied depending on the species of β-glucosidase of *A. niger* has two enzyme with highest pH of 4.2 and 4.8 (41). In general, β-glucosidase activity is greatest in low to neutral pH, depending on the species of microorganism.

CONCLUSION

This study showed the increase in protein content due to proteolytic enzymes activity and loss of dry matter, along with the increase in water content over the fermentation period, contributed to the differences in the enzymatic profile of tempeh incubated for 2 days and 7 days. Based on the microscopic identification results, hyphae and sporangium were found. Meanwhile, the macroscopic identification from the screening and isolation of tempeh fungi on SDA and lactose broth media showed identical characteristics found in the *Rhizopus* species. Then, it was reinforced with molecular identification through PCR amplification of the internal transcribed spacer (ITS) sequence, which showed a DNA fragment with a single band at 700 bp having 99% similarity with *R. microsporus*. Cellulase showed its highest activity in fresh tempeh (2-day incubation) at 0.0911 U/mL at pH 7. Meanwhile, β-glucosidase showed its highest activity in expired tempeh (7-day incubation) at 0.0042 U/mL at pH 5. These findings indicate that fresh tempeh (2-day incubation) is more suitable for consumption as a main food, while expired tempeh (7-day incubation) is more suitable for use as a flavor enhancer in cooking.

ACKNOWLEDGEMENTS

This research was funded by KEMENDIKBUDRISTEK (Fundamental research) under grant no 036/E5/PG.02.00.PL/2024, 323/IT1.B07.1/SPP-LPPM/VI/2024.

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