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IDENTIFICATION OF THE RHIZOPUS SP. FUNGI AS AN ALTERNATIVE LACTIC ACID PRODUCTION SOURCE

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ABSTRACT: The search for eco-friendly alternatives to conventional petroleum-based materials has intensified in an era marked by a growing global awareness of environmental sustainability. This study addresses the critical need for molecular identification and characterization of fungi sourced from a tempeh commercial starter culture for their potential role in fungal-based polymer production. The problem is the limited knowledge and understanding of the genetic composition of these fungi and their suitability for lactic acid (LA) production, which is a crucial component of fungal-based polymer manufacturing. This research examined the tempeh starter culture fungi to identify suitable strains for LA production. The fungi were genotyped by DNA sequencing of the ITS region. The study revealed that the Ragi tempeh commercial starter culture contained only one strain of *Rhizopus* (*R. microsporus*), which was verified through ITS rRNA sequencing with 99.8% similarity to the GenBank database, simplifying control over fungal growth and potentially leading to consistent biomaterial yields. The method employed, involving DNA PCR (and sequencing of the ITS region, proved to be accurate, straightforward, and not excessively labor-intensive. The PCR conditions were as follows: initial denaturation at 98°C for 2 min, followed by 25 cycles of denaturation (98°C for 15 seconds), annealing (60°C for 30 seconds), and elongation (72°C for 30 seconds), with a final extension at 72°C for 10 min. Consequently, the consistent presence of only one *Rhizopus* species in commercial starter cultures of tempeh presents a promising avenue for sustainable biomaterial production, particularly in LA production. The pilot flask setup at 1×10^7 spores/mL was inoculated into 150 mL shake flasks with 1.2 g/mL glucose, incubated at 37°C for 1 to 7 days with 100 rpm shaking, yielding 1.037 g/g after 5 days, demonstrating the feasibility of using this strain for industrial applications.

ABSTRAK: Dalam era yang semakin menekankan kesedaran global terhadap kelestarian alam sekitar, pencarian alternatif mesra alam kepada bahan berasaskan petroleum konvensional semakin giat dijalankan. Kajian ini menangani keperluan kritikal untuk mengenal pasti dan mencirikan kulat secara molekul daripada kultur pemula komersial tempeh bagi potensi penggunaannya dalam penghasilan asid laktik (LA), komponen penting dalam pembuatan polimer berasaskan kulat. Masalah utama yang dibincangkan ialah kekurangan pengetahuan dan pemahaman mengenai komposisi genetik kulat ini serta kesesuaiannya untuk sintesis LA. Kajian ini menumpukan kepada pemeriksaan kulat dari kultur pemula tempeh bagi mengenal pasti strain yang sesuai untuk pengeluaran LA. Penjujukan DNA kawasan *internal transcribed spacer* (ITS) digunakan untuk mengenal pasti genotip kulat tersebut. Hasil kajian menunjukkan bahawa kultur pemula komersial Ragi tempeh hanya mengandungi satu strain sahaja, iaitu *Rhizopus microsporus* (disahkan melalui penjujukan ITS rRNA dengan 99.8% kesamaan dengan pangkalan data GenBank). Kehadiran satu spesies ini memudahkan kawalan pertumbuhan kulat dan berpotensi meningkatkan konsistensi hasil pengeluaran. Kaedah yang digunakan melibatkan PCR DNA dan penjujukan

kawasan ITS, yang terbukti tepat, mudah, serta tidak terlalu memerlukan tenaga kerja yang banyak. Keadaan PCR adalah seperti berikut: penyahdenaturan awal pada suhu 98°C selama 2 minit, diikuti 25 kitaran yang terdiri daripada penyahdenaturan (98°C, 15 saat), pengannealan (60°C, 30 saat), dan pemanjangan (72°C, 30 saat), dengan pemanjangan akhir pada 72°C selama 10 minit. Kehadiran spesies *Rhizopus* yang konsisten dalam kultur pemula tempeh komersial membuka peluang yang menjanjikan untuk pengeluaran asid laktik yang mampan. Penggunaan susunan flask perintis pada suhu 30°C menghasilkan 1.037 g/g selepas 5 hari, membuktikan potensi strain ini untuk aplikasi industri.

KEYWORDS: *DNA sequencing of the ITS region, DNA PCR sequencing, fungal polymer production, lactic acid production, molecular identification of fungi.*

1. INTRODUCTION

There has been growing interest in bio-based polymer production compared to traditional petroleum-based polymers. This shift is primarily driven by bio-based polymers' renewable and eco-friendly nature, particularly considering the declining petroleum reserves [1, 2]. Among the prominent bio-based polymers, polylactic acid (PLA), often called bioplastic, has garnered significant attention. Polylactic acid (PLA), commonly called bioplastic, has attracted considerable attention among these polymers. Lactic acid (LA), a key precursor of PLA, is produced through fermentation and exists as two enantiomers, D-LA and L-LA. While D-LAs are considered hazardous, L-LAs are deemed safe and widely used in the food and beverage industries [3, 4].

Both lactic acid bacteria (LAB) and fungi produce LA. Still, fungi are preferred for biomaterial production because of their adaptability to a wide range of renewable substrates, such as sugar and starch, compared with LAB [4]. Additionally, purification technology for fungal-derived LA is more mature and cost-effective. Fungi are abundant, easy to cultivate, and require minimal nutrients. Among the various fungal species capable of LA production, *Rhizopus sp*., including *R. oryzae*, *R. arrhizus*, and *R. microsporus*, have emerged as promising biomaterial sources [5].

Several studies have demonstrated the ability of *Rhizopus* spp. to produce LA. For instance, [6] demonstrated how well *R. oryzae* produces LA using a range of agro-industrial residues as substrates. Similarly, [7] observed that *R. arrhizus* produced a high yield and productivity of LA when the fermentation conditions were optimized. Using R. microsporus and corn stover as substrates [8] demonstrated high LA production. Furthermore, [9] highlighted the potential of food waste as a cost-effective substrate for LA production in *R. oryzae*. A study by [10] successfully demonstrated LA production using *R. arrhizus* with cassava pulp as the substrate (Table 1). In this context, high production refers to efficiently generating large amounts of LA. This is typically indicated by high yield (≥1.0 g LA/g substrate), fast production rate, and high final concentration in the fermentation broth.

Despite these advances, the potential of fungi from tempeh starter cultures to produce LA remains to be explored. Traditional identification methods have primarily focused on LAB, overlooking the efficiency and sustainability of the fungal species. DNA PCR and sequencing are the key molecular techniques used for genotyping and studying fungal sources [11-13]. This study aimed to bridge this gap by focusing on the molecular identification and characterization of fungi derived from tempeh starter culture. Specifically, the internal transcribed spacer (ITS) region, a widely used fungal DNA barcode, was analyzed to achieve precise species identification and to characterize inter- and intraspecific variations. The findings of this study could contribute to a more sustainable and efficient bio-based polymer

production. The LA generation route begins with glucose traveling via glycolysis, which breaks it down into pyruvic acid, while producing ATP and NADH. Lactate dehydrogenase (LDH) converts pyruvic acid to LA under anaerobic conditions by oxidizing NADH to NAD^+, a necessary step for glycolysis. Microorganisms such as *Lactobacillus* and *Rhizopus sp.* efficiently ferment sugars from various renewable substrates, facilitating this process [14]. The ability of fungi to exploit lignocellulosic biomass and other agro-industrial leftovers makes them a possible source for long-term LA production [15].

Table 1. Recent Similar Studies on Fungal-Based Lactic Acid Production

Fungal Species	Substrate Used	Yield (g/g)	Key Findings	Ref
R. oryzae	Agro-industrial residues	0.9	Efficient conversion of agro-industrial residues to LA	[6]
R. arrhizus	Optimized fermentation media	1.2	High yield and productivity under optimized conditions	[7]
R. microsporus	Corn stover	1.05	High LA production using lignocellulosic biomass	[8]
R. oryzae	Food waste	1.15	Potential of food waste as a cost-effective LA substrate	[9]
R. arrhizus	Cassava pulp	1.0	Efficient LA production from cassava pulp	[10]

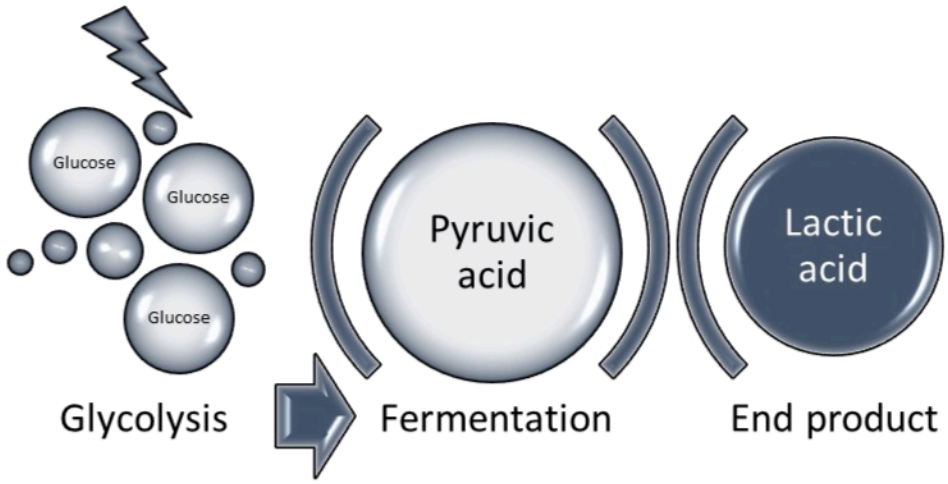

Figure 1. LA Production Pathway

2. MATERIALS AND METHODS

2.1. Yeast growth and genomic DNA extraction

The Ragi tempeh commercial starter culture, sourced from PT Aneka Fermentasi Industri in Indonesia and known to contain *Rhizopus sp.,* was cultured, isolated, and cultivated on potato dextrose agar (PDA) at an incubation temperature of 37ºC for a period of 7 days. Subsequently, *Rhizopus* growth on the agar was excised (0.5 cm x 0.5 cm) using a sterilized blade and transferred into 1.5 mL centrifuge tubes. This process was performed in triplicate to collect the yeast samples. Genomic DNA (gDNA) of the *Rhizopus* isolates was extracted using the Presto™ Mini gDNA Yeast Kit (Geneaid, GBYB100), followed by PCR amplification of the Internal Transcribed Spacer (ITS) gene using the universal primers ITS1 and ITS4.

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2.2. Application of the ITS region

Amplification of the fungal ITS gene was accomplished using a pair of universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Each reaction mixture, totaling 25 µL in volume, consisted of purified gDNA, 0.5 pmol DNA template, deoxynucleotide triphosphates (dNTPs, 200 µM each), thermostable DNA polymerase (0.5 U), PCR buffer, and dH_2O. The PCR procedure included an initial denaturation cycle at 98°C for 2 min followed by 25 cycles of denaturation at 98°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The final extension step was performed at 72°C for 10 min. The PCR products were visualized on a 1% TAE agarose gel at 100 V for 60 minutes [17]. The amplified PCR products were purified and subjected to bidirectional sequencing using M13F (-20) and M13R-pUC (-26) primers and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing was performed by First BASE Laboratories (Apical Scientific Sdn. Bhd.).

2.3. BLAST and phylogenetic tree of the ITS region

The resulting ITS sequences (~700 bp) were analyzed and compared against the NCBI nucleotide database (nr/nt) using BLAST for fungal identification, accessible on the National Centre for Biotechnology Information (NCBI) website [18]. Neighbor-joining analysis was conducted to create an unrooted phylogenetic tree. This analysis was performed using the NCBI BLAST tree method available on the NCBI website [18].

2.4. Culture *Rhizopu*s in the fermentation medium

The fungi were cultured on potato dextrose agar (PDA) at 37°C for one week in Petri dishes with replicates. After sporulation and proliferation, 10 mL of distilled water was poured aseptically onto each agar plate, which was then scraped to release spores. The spore suspension was centrifuged for 10 min at 4,000 rpm, washed, and resuspended in 1 mL of distilled water. Then, a 1 mL spore suspension containing approximately 1 × 107 spores/mL was utilized to provide a constant quantity of spores for each experiment. The spores were inoculated and grown in each medium within a shake flask (150 mL) containing 1.2 g/mL glucose at 37°C, for 1–7 days, and at an agitation speed of 100 rpm in an incubator shaker. Fungal spore concentration was determined using a hemocytometer (Neubauer improved chamber) under a light microscope (40× magnification). After culturing the fungi on potato dextrose agar (PDA) at 37°C for one week, spores were harvested by adding 10 mL of sterile distilled water to the petri dish and scraping the surface. The spore suspension was centrifuged at 4,000 rpm for 10 min, washed, and resuspended in 1 mL sterile distilled water. A 10 µL aliquot of the well-mixed spore suspension was loaded onto the hemocytometer, and spores were counted in four large squares of the grid. The spore concentration was calculated using the following formula:

$$Spores\ concentration\ \left(\frac{Spores}{mL}\right) = \frac{Average\ spore\ count\ per\ square \times Dilution\ factor \times 10^4}{Volume\ of\ counted\ samples\ (mL)} \quad (1)$$

The final concentration was adjusted to 1×10^7 spores/mL by dilution with sterile distilled water or concentrating via centrifugation before inoculation into the fermentation medium.

2.5. LA Concentration Analysis

The LA concentration was determined using a colorimetric assay with iron (III) chloride ($FeCl_3$) at a wavelength of 540 nm using a DR5000™ Hach UV-VIS spectrophotometer. A stock LA solution with a concentration of 89 g/L was prepared by dissolving 1.2 g/mL of LA (89% purity) in distilled water to prepare the LA standard curve. Serial two-fold dilutions were

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performed to generate six LA solutions with concentrations ranging from 0 to 5 wt.%. A 0.2% $FeCl_3$ nanoparticle solution was prepared by dissolving 0.2 g of $FeCl_3$ nanoparticle in a 100 mL volumetric flask and diluting it with distilled water to the required volume. The solution was stored at 25°C (room temperature) and swirled until thoroughly mixed. For the absorbance measurements, 2 mL of 0.2% $FeCl_3$ solution was mixed with 50 µL of each LA standard solution. These solutions' optical density (O.D.)/absorbance ratio was measured at 540 nm using a UV-VIS spectrophotometer, and a standard calibration curve was constructed. The equation of the standard curve was determined as follows:

$$y = 0.9939x \tag{2}$$

where y represents the absorbance value (a.u.) and x is the LA concentration (wt.%). The coefficient of determination for the calibration curve was $R^2 = 0.99$, ensuring the accuracy of the concentration calculations. To determine the LA concentration in the fermentation samples, 2 mL of 0.2% $FeCl_3$ solution was mixed with 50 µL of the experimental sample, and the absorbance was measured at 540 nm. The LA concentration was calculated using Eq. (2) based on a plotted standard curve.

3. RESULTS AND DISCUSSION

3.1. Yeast ITS Region PCR Amplicon Result

DNA extraction was successfully performed. Assessment of DNA purity through spectrophotometric measurements within the 240–260 nm range indicated a ratio greater than 1.8, signifying good quality [19]. Moreover, amplification of the *Rhizopus* isolate ITS amplicon was successfully achieved, resulting in a single band of approximately 750 bp (Fig. 2). Notably, a positive control band appeared at approximately 700 bp. This variation is anticipated because the positive control employed a universal ITS, which may have slight differences in the length of its ITS region compared to that of *Rhizopus sp.*

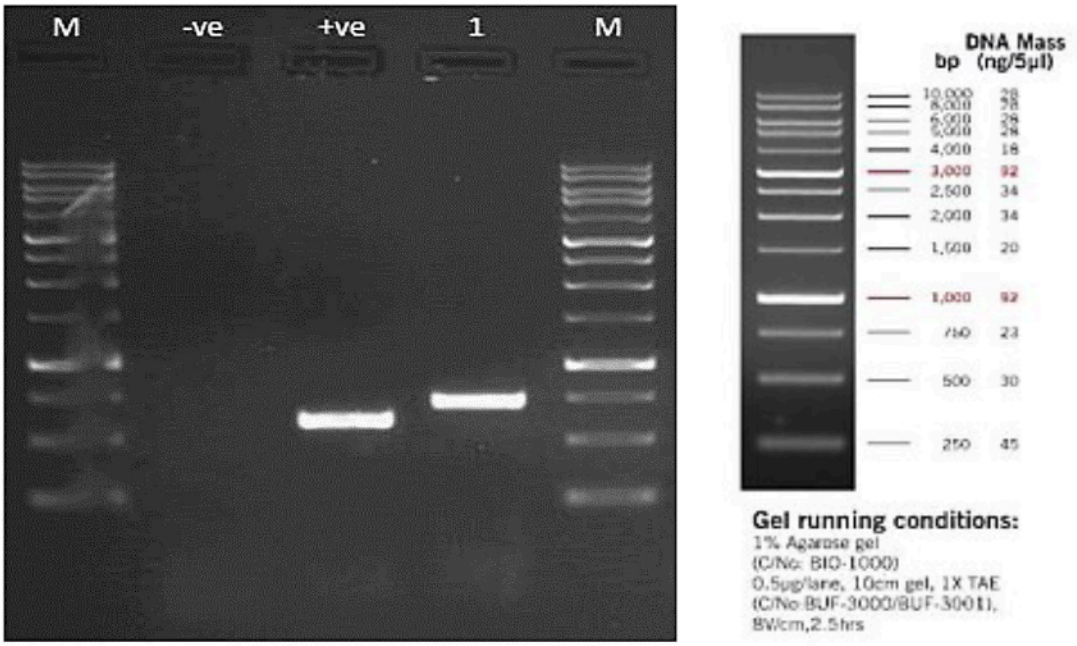

Figure 2. Gel electrophoresis image showing PCR amplicons. M, DNA ladder; −ve, no template control; +ve, positive control (purified plasmid with ITS region insert, 1 ng); 1: Rhizopus isolate from Ragi tempeh.

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3.2. DNA Sequencing and BLAST Analysis

Purified ITS amplicons from the *Rhizopus* isolate were successfully processed and sequenced. The sequencing outcomes obtained using the ITS region's forward and reverse primers yielded pure, high-quality, and easily readable DNA sequences (Fig. 3. Table 2 provides the complete ITS DNA sequence assembled from *Rhizopus* isolate sequencing. Subsequently, the ITS DNA sequence (Table 2) was compared to the GenBank database using BLASTN [18]. The phylogenetic tree results, illustrated in Figure 3, revealed a single cluster, all indicating the *Rhizopus microsporus* strain.

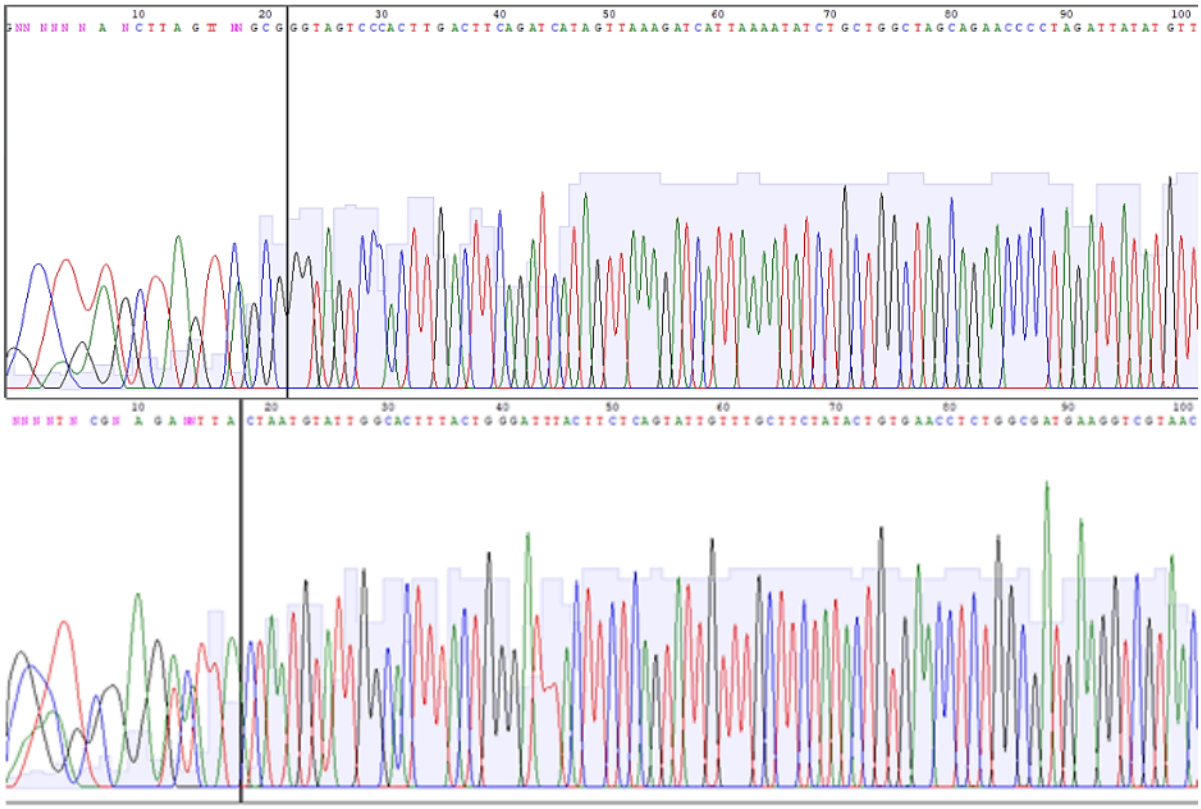

Figure 3. DNA sequence of the first 100 bp obtained from the Ragi tempeh *Rhizopus* isolate. Amplicons were obtained using an ITS reverse primer. The black line indicates the start of a good, readable sequence.

Table 3. ITS sequence identified from Ragi tempeh *Rhizopus* isolate.

Amplicon Sample	ITS Sequence Identify
Ragi tempeh *Rhizopus* isolate	CCAGTTCCGTAGGTGAACCTGCGGAAGGATCATTAACTAATGTATTGGCACTTTA CTGGGATTTACTTCTCAGTATTGTTTGCTTCTATACTGTGAACCTCTGGCGATGAA GGTCGTAACTGACCTTCGGGAGAGACTCAGGACATATAGGCTATAATGGGTAGG CCTGTTCTGGGGTTTGATCGATGCCAATCAGGATTACCTTTCTTCCTTTGGGAAGG AAGGTGCCTGGTACCCTTTACCATATACCATGAATTCAGAATTGAAAGTATAATA TAATAACAACTTTTAACAATGGATCTCTTGGTTCTCGCATCGATGAAGAACGTAG CAAAGTGCGATAACTAGTGTGAATTGCATATTCGTGAATCATCGAGTCTTTGAAC GCAGCTTGCACTCTATGGATCTTCTATAGAGTACGCTTGCTTCAGTATCATAACCA ACCCACACATAAAATTTATTTTATGTGGTGATGGACAAGCTCGGTTAAATTTAAT TATTATACCGATTGTCTAAAATACAGCCTCTTTGTAATTTTCATTAAATTACGAAC TACCTAGCCATCGTGCTTTTTTGGTCCAACCAAAAAACATATAATCTAGGGGTTCT GCTAGCCAGCAGATATTTTAATGATCTTTAACTATGATCTGAAGTCAAGTGGGAC TACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAGTCAT

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This study successfully identified a specific *Rhizopus* strain found in the Ragi tempeh commercial starter culture. According to the latest *Rhizopus* taxonomy, our findings strongly suggest the exclusive presence of a single *Rhizopus* species within the Ragi Tempeh commercial starter culture. The ITS rRNA sequence obtained from the Ragi Tempeh commercial starter culture was confirmed to belong to the species *R. microsporus.* The obtained ITS rRNA sequence from the Ragi tempeh commercial starter culture was analyzed using BLAST, revealing 99.8% similarity with *R. microsporus* strain. The highest identity matches confirmed a 627 bp sequence length, aligning with reference ITS sequences for these species. The substantial genetic similarity supports the isolate classification as *Rhizopus microsporus*, as it shares identical ITS rRNA sequences with known *R. microsporus* strains. Conserved ITS regions further validate this identification, confirming its role as a dominant species in tempeh fermentation.

This study also highlighted the effectiveness of ITS sequences as DNA barcodes for identifying fungal species. Identifying a singular *Rhizopus sp.* species was well-founded, considering that tempeh commercial starter cultures have long been recognized to contain only one *Rhizopus sp*. strain. The reduction in *Rhizopus* diversity in tempeh, linked to the widespread use of commercial tempeh starters in Indonesia since the early 2000s, is evident [20]. This phenomenon was clearly illustrated in the phylogenetic analysis of the ITS sequence results obtained from *Rhizopus* isolated from a commercial starter culture (see Figure 4). ITS sequences, which are shared by numerous fungal kingdoms, including *Rhizopus sp.,* have been extensively documented and continue to be updated [13].

Adopting DNA PCR and sequencing of the ITS region proved accurate and user-friendly, significantly reducing the labor involved compared to alternative methods, such as microscopic morphological characterization or random amplified polymorphic DNA analysis [21]. This advantage holds particular value at the industrial level, especially during quality control assessments of *Rhizopus* growth.

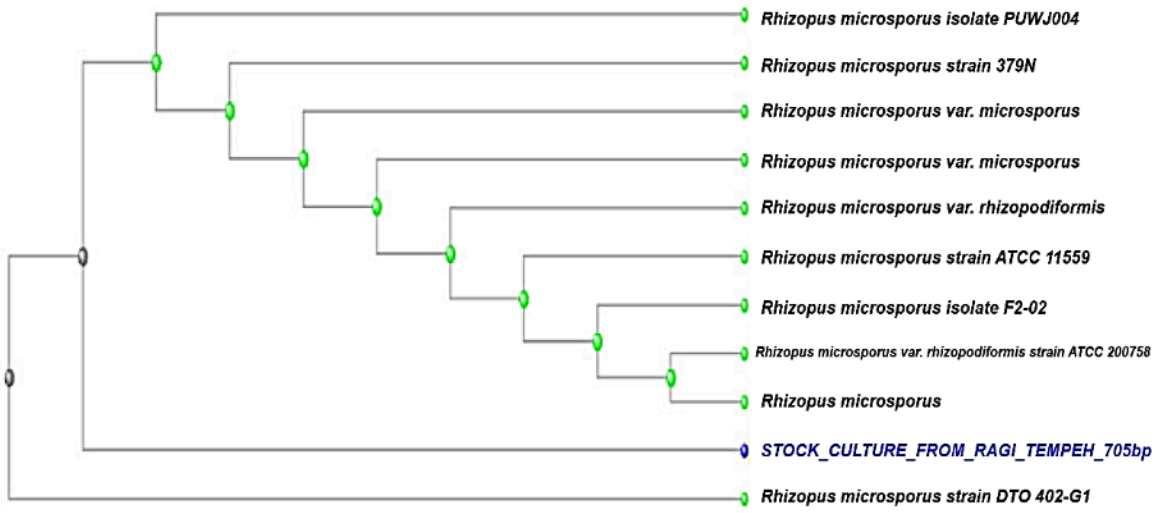

Figure 4. The BLAST results of the query sequence to the most similar biological sequences are available in the NCBI database.

The outcomes of the molecular identification and the resulting phylogenetic tree analysis, based on the ITS region of the isolated *Rhizopus* strain from the Ragi tempeh, confirm that the prolonged use of tempeh commercial starter cultures has led to the decline of traditional *Rhizopus* strains used in tempeh production [22] Traditionally, the inoculum for tempeh fermentation, known as "Usar," is typically derived from the leaves of *Hibiscus tiliaceaus* [23].

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It has been documented that when Usar is the source of the initial tempeh inoculum, multiple strains of various *Rhizopus* species, such as *R. delamar, R. oryzae*, and *R. arrhizus*, have been identified [22]. The reduction in *Rhizopus* species diversity within tempeh could potentially affect its status as a healthy food product, although it may find applications in other industries.

Nevertheless, a single *Rhizopus sp.* species within the Ragi Tempeh commercial starter culture could offer advantages for LA production. This is because various *Rhizopus* species have been reported to exhibit distinct nutritional preferences for their growth [22]. Consequently, when fungi with complex nutritional requirements are used, it becomes challenging to streamline LA production because multiple factors, such as nitrogen substrate, temperature, and pH, need to be meticulously controlled. Furthermore, LA production efficiency is highly susceptible to fluctuations when different fungi exhibit varying growth rates [1]. Notably, interest in this field declined from 2017 onwards [23]. In contrast, only one strain of *Rhizopus* (*R. microsporus*) within the Ragi Tempeh commercial starter culture simplifies the control of fungal growth, potentially leading to consistent biomaterial yields. Fig. 5 shows the species image under ten times magnification of the light contrast microscope.

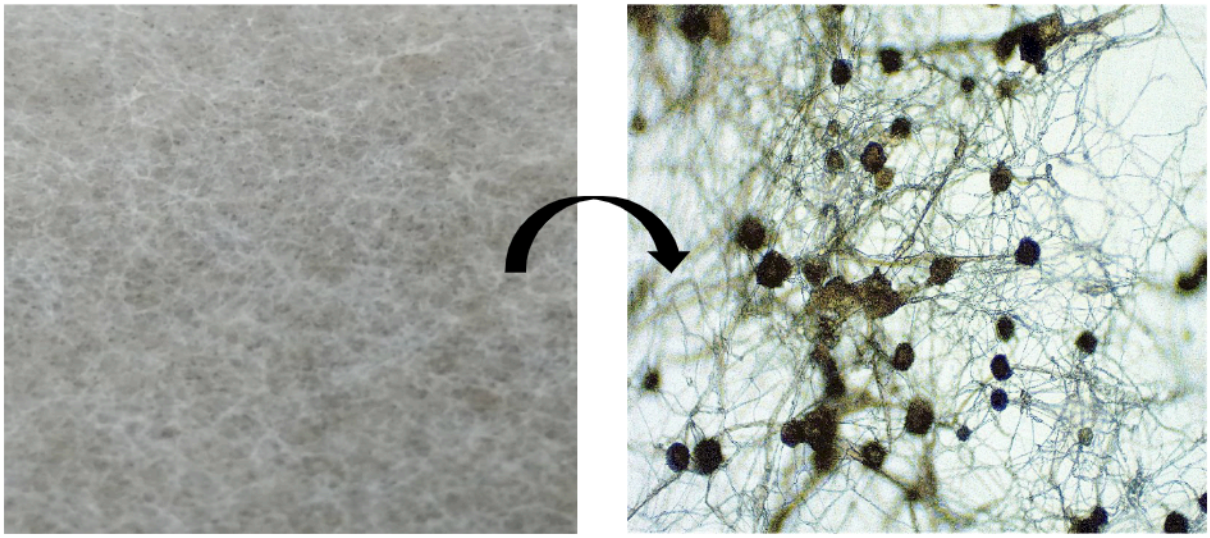

Figure 5. *R. microsporus* fungi under the naked eye on PDA. The image on the right is the zoom view of the species under 10x magnification of the light microscope.

3.3. LA Yield from the identified strain

To prove the effect of fungal strain cell metabolism on the production of LA, fermentation reactions using glucose yielded LA. Thus, the experimental yield was 1.037 g/g after 5 days in the pilot flask size setup at 30 °C, as shown in Figure 6. The maximum LA yield showed significantly higher results than a study done by [24], which recorded 0.95 g/g LA yield, respectively.

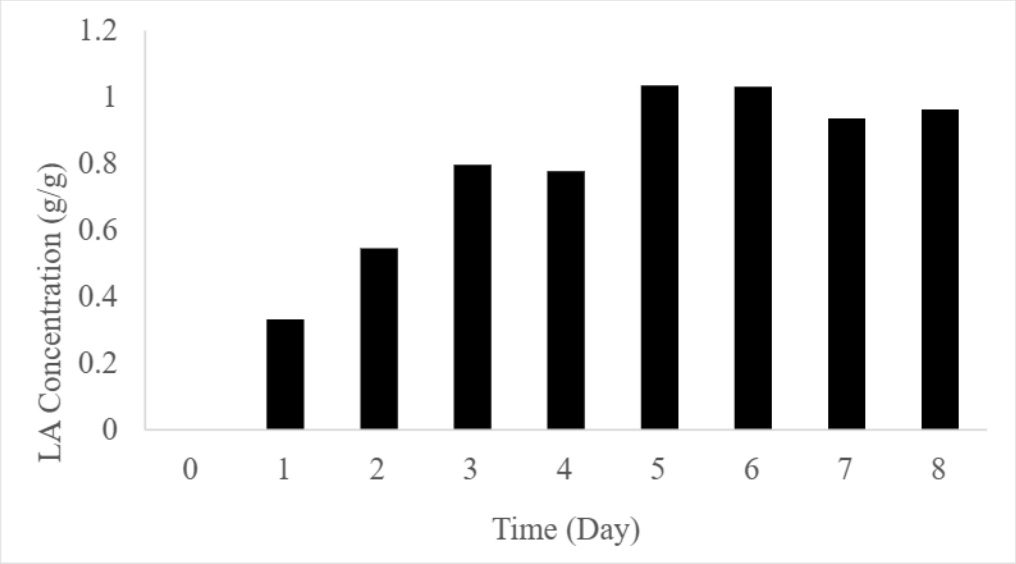

Figure 6. LA concentration produced by *Rhizopus microsporus.* over 7 days of incubation time

4. CONCLUSIONS

Molecular analysis confirmed that the *Rhizopus* strain isolated from the Ragi Tempeh commercial starter culture falls under the classification of *R. microsporus.* This molecular identification method, which involves DNA PCR and ITS region sequencing, has proven precise, straightforward, and efficient. Moreover, the consistent presence of a single *Rhizopus* species within tempeh commercial starter cultures offers a promising opportunity for venturing into biomaterial production. Specifically, it presents a potential avenue for LA production using the tempeh commercial starter culture, as the LA yield was 1.037 g/g.

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