Journal of Biodiversity and Maritime Study
Vol. 2, No. 1, Hlm 1-8

APPLICATION OF PROTEINASE K TO FACILITATE FUNGAL CELL LYSIS IN COMMERCIAL SILICA COLUMN-BASED DNA ISOLATION KIT

Fabian Rizky Fathurahman[1)*], Patrick Pascalis Galih Seto Baskara[2)], Saskia Sausan Ramadhana Sally[2)], Aisha Giefira Nur Fatimah[2)], Ana Harisatien Hamidah[2)], Lifiyana Eka Khumaira[2)], Farah Ispramudita Septiyanti[3)], Oviliana Laurentia Saputri[3)], Elda Zaelita Nurul Raizma[3)], Tanaya Aryo Jalu Bimantoro[3)], Elroy Bryan Wilson Sijabat[3)], Azka Kamila Shalih[3)], Galang Anahatta Hidayat[3)],Taruna Dwi Satwika[3)], Yuriza Eshananda[3)*]

[1] *Master's Program in Biology, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Central Java, Indonesia*
[2] *Microbiology Study Program, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Central Java, Indonesia*
[3] *Biology Study Program, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Central Java, Indonesia*

*E-mail: fabian.rizky.fathurahman@gmail.com

ABSTRACT

Efficient isolation of high-quality genomic DNA is essential for molecular analyzes such as PCR and sequencing in fungal studies. However, the rigid cell walls of mold, composed predominantly of chitin and β-glucans, pose a significant challenge to standard lysis protocols. This study investigates the integration of Proteinase K into a commercial silica column-based DNA isolation kit to enhance lysis efficiency and DNA recovery from mold cells. Four unidentified fungal isolates were subjected to DNA isolation using a modified protocol incorporating Proteinase K as treatment during the lysis step. The fungal DNA was quantified spectrophotometrically and evaluated via Polymerase Chain Reaction (PCR) using fungal-specific barcoding of the Internal Transcribed Spacer (ITS) region, amplified by ITS 5 and ITS 4 primers. The findings demonstrate that the inclusion of Proteinase K along with incubation time significantly improves cell lysis, leading to increased DNA yield and successful PCR amplification of the ITS region across all tested isolates. These results reveal the utility of Proteinase K as an effective, cost-efficient enzymatic supplement to conventional DNA isolation workflows of fungi, especially when working with structurally complex cell wall mold taxa.

Keywords: Proteinase K, fungi, DNA isolation

ABSTRAK

Isolasi DNA genom berkualitas tinggi yang efisien sangat penting dalam analisis molekuler seperti Polymerase Chain Reaction (PCR) dan sekuensing dalam penelitian berkaitan dengan Fungi (jamur). Namun demikian, dinding sel jamur yang rigid, yang sebagian besar terdiri dari kitin dan β-glukan menimbulkan tantangan yang signifikan terhadap protokol lisis standar. Penelitian ini bertujuan untuk mengetahui integrasi Proteinase K ke dalam kit isolasi DNA berbasis kolom silika komersial untuk meningkatkan efisiensi lisis dan pemulihan DNA dari sel jamur. Empat isolat jamur yang belum teridentifikasi dilakukan isolasi DNA menggunakan protokol yang dimodifikasi dengan menggabungkan Proteinase K sebagai perlakuan selama langkah lisis. DNA jamur diukur menggunakan metode spektrofotometri dan dievaluasi melalui serangkaian proses Polymerase Chain Reaction (PCR) berdasarkan penanda spesifik DNA jamur dari daerah Internal Transcribed Spacer (ITS), yang diamplifikasi melalui primer ITS 5 dan ITS 4. Hasil penelitian menunjukkan bahwa penambahan Proteinase K dan waktu inkubasi secara signifikan meningkatkan lisis sel, yang menyebabkan

peningkatan konsentrasi DNA dan keberhasilan amplifikasi PCR dari daerah ITS pada semua isolat yang diujikan. Hasil tersebut menunjukka fungsi Proteinase K sebagai suplemen enzimatik yang efektif dan hemat biaya dalam tahapan isolasi DNA jamur secara konvensional, terutama saat bekerja dengan kelompok jamur dengan dinding sel yang kompleks secara struktural.

Kata Kunci: Proteinase K, fungsi, isolasi DNA

1. INTRODUCTION

Fungi are eukaryotic organisms that play essential ecological and industrial roles, ranging from organic matter decomposition and symbiosis with plants to applications in food production, pharmaceuticals, and biotechnology (Corbu *et al*., 2023; Khan *et al*., 2022). The increasing relevance of fungi in diverse research areas has driven the demand for molecular approaches such as DNA barcoding, phylogenetics, and metagenomics to elucidate species identity, evolutionary history, and functional potential (Yang *et al*., 2025). A key requirement for these molecular approaches is the isolation of high-quality genomic DNA, which must be intact, free of inhibitors, and amplifiable to ensure reliable downstream analyses such as PCR, sequencing, and cloning (Luna *et al*., 2024; Khan *et al*., 2023).

However, the isolation of DNA from fungal cells remains technically challenging due to the unique composition and rigidity of the mold cell wall, which typically includes chitin, β-glucans, and other polysaccharides (Ghassemi *et al*., 2021). These structural features confer resistance to standard lysis buffers and enzymatic treatments, often leading to poor DNA yield and quality (Al-Madboly *et al*., 2024). Mechanical disruption methods such as bead beating or grinding in liquid nitrogen can improve lysis efficiency, but they are labour-intensive and not always compatible with high-throughput workflows (Langsiri *et al*., 2025; Duong & Lee, 2023).

To address these limitations, many laboratories adopt silica membrane-based commercial DNA isolation kits due to their convenience, consistency, and suitability for a wide range of sample types (Goyani & Mukhopadhyaya, 2023). These kits rely on the selective binding of DNA to a silica membrane in the presence of chaotropic salts, followed by washing steps to remove contaminants and elution of purified DNA (Barcenilla *et al*., 2024). Despite their widespread use, the standard protocols provided in these commercial kits are typically optimised for bacterial, animal, or plant cells, and may not be sufficiently robust to break down the rigid cell walls of fungi (Fathurahman *et al*., 2025; Galliano *et al*., 2021). Consequently, protocol modifications are often necessary to optimise DNA yield and purity from fungi samples.

While chitinase has been recognised for its ability to hydrolyse chitin, a major structural component of mold cell walls, its use in DNA isolation protocols is often limited by several practical constraints (Kovács-Simon & Fones, 2024). Chitinase is typically expensive, sensitive to storage conditions, and may require prolonged incubation times or specific pH and temperature settings to function optimally (Oyeleye & Normi, 2018). In contrast, Proteinase K is a broad-spectrum serine protease that is more stable, cost-effective, and easier to integrate into standard lysis protocols without requiring extensive optimisation. It not only facilitates the degradation of structural proteins that contribute to cell wall rigidity but also inactivates nucleases that can compromise DNA integrity (Panilo & Rice, 2021). Moreover, Proteinase K is compatible with a wide range of commercial silica-based isolation kits and has consistently demonstrated effectiveness in improving DNA yield and quality across diverse sample types, including fungi with tough cell walls (Ruggieri *et al*., 2016; Kalendar *et al*., 2021; Jillwin *et al*., 2021). Therefore, Proteinase K is favoured in this study due to its versatility, reliability, and ease of use compared to chitinase for fungal DNA isolation workflows.

Accordingly, this study aims to assess the effectiveness of integrating Proteinase K into the lysis step of a commercial silica-based DNA isolation protocol specifically for fungal samples. The primary objective is to determine whether the enzymatic treatment can facilitate more efficient cell wall disruption and lead to the recovery of high-quality genomic DNA suitable for molecular applications. The quality of the isolated DNA will be evaluated based on its ability to serve as a reliable template in PCR amplification, providing insights into the practical utility of Proteinase K as an additional suitable protocol for fungal DNA isolation.

2. RESEARCH METHODOLOGY

2. 1. Tools and Materials

This study was conducted in the Microbiology Laboratory and the Genetics and Molecular Laboratory of the Faculty of Biology, as well as in the Research Laboratory within Universitas Jenderal Soedirman, Purwokerto, Central Java, from March until May 2025. The equipment used in this study included micropipettes (Eppendorf), sterile tips (Axygen), 1.5 mL and 0.2 mL microcentrifuge tubes (Axygen), a vortex mixer (Accu Biosystems), a microcentrifuge (DLab), a thermomixer (Eppendorf), microtube racks, a UV-Vis NanoDrop spectrophotometer (Implen), a Mupid-EXu electrophoresis apparatus (Takara), a Primus 25 thermal cycler (Peqlab) and a benchtop UV Transiluminator MultiDoc-it Digital Imaging System (UVP).

The materials used were multiple mold isolates obtained from the collection of the Mycology Laboratory, Faculty of Biology, Universitas Jenderal Soedirman. (**Table 1**), Proteinase K enzyme (20 mg/mL) (Thermo-Scientific), potato dextrose broth (PDB) media, Wizard® SV Genomic DNA Purification System (Promega), nuclease-free water (Promega), agarose powder for gel electrophoresis (NzyTech), FluoroVue™ Nucleic Acid Gel Stain (Smobio), 1kb DNA ladder (Smobio), 1x TAE electrophoresis buffer (Thermo-Scientific), MyTaq HS 2× Mastermix (Meridian Bioscience), ITS 4, and ITS 5 primers (IDT). Sequences of the primers were: ITS5 (forward): 5′-GGAAGTAAAAGTCGTAACAAGG-3′ and ITS4 (reverse): 5′-TCCTCCGCTTATTGATATGC-3′ (White, 1990).

Table 1. List of mold isolates used in this Study.

No	Isolate Code	Species
1	11A41	Uncharacterized
2	11A42	Uncharacterized
3	11D2	Uncharacterized
4	11D3	Uncharacterized

2.2. DNA Isolation

Mold cultures were grown in liquid potato dextrose broth (PDB) for 4 days at room temperature with shaking at 120 rpm. After incubation, mold biomass was harvested by centrifugation at 10,000 × g for 5 minutes at 4°C, and the supernatant was discarded. The resulting mycelial pellet (approximately 50–100 mg wet weight) was transferred into a sterile 1.5 mL microcentrifuge tube. Genomic DNA was then isolated using the Wizard® SV Genomic DNA Purification System (Promega), with a modified cell lysis step. The mycelial pellet was resuspended by pipetting in the kit-provided lysis buffer, and 20 µL of Proteinase K (20 mg/mL) (Thermo-Scientific) was added. The suspension was incubated overnight at 56 °C with agitation at 1000 rpm in a thermomixer to ensure efficient cell wall degradation. Following incubation, the lysate was briefly centrifuged at 13,000 × g for 1 minute to pellet debris. The clear supernatant was transferred to a new microcentrifuge tube, mixed with the binding buffer according to the manufacturer's protocol, and loaded onto the silica column. Washing and elution steps were performed as described in the kit manual. DNA was eluted in nuclease-free water (Promega) and stored at −20 °C.

2. 3. DNA Quantification

DNA quantification was carried out using a UV-Vis Nanodrop spectrophotometer (Implen). The instrument was first initialised, and the pedestal was blanked using 1–2 µL of nuclease-free water (Promega). After blanking, 1–2 µL of the DNA sample was loaded onto the measurement pedestal, and the absorbance was measured at 260 nm and 280 nm to determine DNA concentration and purity. The A260/A280 ratio was used as an indicator of DNA purity, with a ratio of approximately 1.8 indicating high-quality DNA. After each measurement, the pedestal surfaces were cleaned using a lint-free tissue to prevent cross-contamination between samples.

2. 4. Amplification of the ITS Marker

PCR amplification was performed using MyTaq HS 2× Mastermix (Meridian Bioscience) in a final reaction volume of 12.5 µL. Reaction mixture consisted of 6.25 µL MyTaq HS 2× Mastermix, 0.5 µL ITS4 primer (10 µM), 0.5 µL ITS5 primer (10 µM), 1.0 µL template DNA, and 4.25 µL nuclease-free water (Promega). PCR thermal cycling conditions were as follows: initial denaturation at 95 °C for 1 minutes; 35 cycles of denaturation at 95 °C for 15 seconds, annealing at 55.5 °C for 30 seconds, and extension at 72 °C for 30 seconds; followed by final extension at 72 °C for 5 minutes, and hold at 4 °C. PCR products were analyzed by gel electrophoresis using 1.2% agarose gel prepared with 1x TAE electrophoresis buffer and stained using FluoroVue™ Nucleic Acid Gel Stain (Smobio). Electrophoresis was conducted in the Mupid-EXu system (Takara) at 100 volts for 25 minutes. DNA bands were visualised under a UV transilluminator.

3. RESULTS AND DISCUSSION

Accurate assessment of DNA quantity and purity is a crucial step following the isolation process, as it determines the suitability of the isolated nucleic acids for downstream molecular applications. In this study, the quality of DNA isolated from mold samples was evaluated using UV-Vis Nanodrop spectrophotometry, focusing on absorbance values at specific wavelengths to assess both concentration and potential contamination.

Table 2. Results of DNA quantification of all samples as determined by UV-Vis Nanodrop Spectrophotometry.

Sample	A230	A260	A280	A320	A260/A280	A260/A230	DNA Concentration (ng/µL)
11A41	2.281	0.404	0.229	0.057	2.017	0.156	174
11A42	1.873	0.753	0.520	0.278	1.963	0.298	238
11D2	1.983	0.136	0.085	0.026	1.864	0.056	55
11D3	2.286	0.363	0.188	0.022	2.054	0.151	171

DNA quantification results obtained through UV-Vis Nanodrop spectrophotometry (**Table 2**), demonstrate notable variation in both concentration and purity across the fungal samples. Sample of code 11A42 exhibited the highest DNA yield at 238 ng/µL, whereas sample 11D2 yielded the lowest at only 55 ng/µL. These discrepancies could be attributed to multiple factors, including differences in the structural composition of the mold cell walls, efficiency of lysis during the Proteinase K treatment, or variations in DNA binding and elution efficiency within the silica column-based purification kits (Kumari et al., 2023; Minkner *et al*., 2022). Moreover, Sankar *et al*., (2025) also stated that physical factors such as sample homogenization, agitation during lysis, or incomplete removal of cellular debris might have contributed to inconsistent recovery.

Despite the variability observed among samples, the relatively high DNA concentrations, particularly in isolates such as 11A42 (238 ng/µL) and 11A41 (174 ng/µL), indicate the overall effectiveness of the modified protocol incorporating Proteinase K during the lysis step. The enzymatic activity of Proteinase K likely enhanced the breakdown of proteinaceous and structural components within the rigid mold cell wall matrix, facilitating more efficient release of genomic DNA into solution. This improvement in lysis efficiency is reflected in the consistently detectable and quantifiable DNA yields across all isolates, even in samples with lower purity ratios. These findings support previous studies (e.g., Saravanan *et al*., 2021; Panilo & Rice, 2021) that highlight Proteinase K as a highly effective, broad-spectrum protease capable of improving DNA recovery, especially from challenging mold cell walls. Therefore, the inclusion of Proteinase K can be considered a key factor contributing to the success of the DNA isolation process in this study.

The optimal DNA purity ranges from 1.8 to 2.0 nm, determined by the absorbance ratio of 260 and 280 nm. The comparison of DNA purity with these ratios' range indicates that the purity requirements necessary for molecular analysis have been fulfilled (Saputra et al., 2024). Purity assessment based on A260/A280 ratios reveals that most samples fall within or slightly above the

acceptable range for DNA (~1.8), with values such as 2.017 (11A41) and 2.054 (11D3) suggesting potential RNA carryover or, less likely, slight overestimation due to hyperchromicity (Russel & Sambrook, 2001). These elevated values, while not necessarily detrimental, indicate that RNase treatment might be considered in future protocols to improve DNA specificity.

More spesifically, the A260/A230 ratios across all samples are substantially lower than the optimal benchmark of 2.0–2.2 (Lucena-Aguilar *et al*., 2016), ranging from 0.056 to 0.298. Such low values strongly suggest the presence of residual contaminants such as polysaccharides, phenolic compounds, chaotropic salts, or even remnants of Proteinase K and other buffer components. These substances are known to absorb at 230 nm and can inhibit downstream enzymatic processes like PCR or sequencing if not sufficiently removed (Al-Griw *et al*., 2017).

However, the most accurate indicator of DNA quality is functioning in the intended downstream application, even though spectral profiles and purity ratios are important indicators of sample quality (Matlock, 2015). In this study, we found that our sample did not meet of standard criteria of "pure DNA" mentioned above. Thus, the isolated DNA was further assessed for its suitability in downstream applications through Polymerase Chain Reaction (PCR). This step was essential to determine whether the residual contaminants interfered with enzymatic activity or if the DNA quality, despite its suboptimal purity, remained adequate to support successful amplification. The PCR amplification results using ITS primers are presented in **Figure 1**.

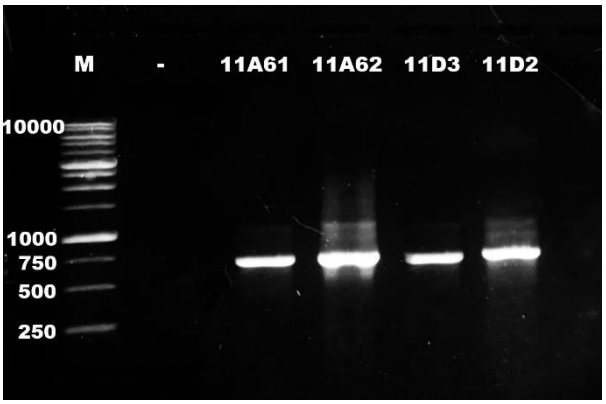

Figure 1. Gel electrophoresis visualisation of ITS marker amplification through PCR (M: 1 kb DNA ladder, -: Negative control).

The electrophoresis profile presented in Figure 1 confirms successful amplification of the internal transcribed spacer (ITS) region across all examined mold DNA samples: 11A61, 11A62, 11D3, and 11D2. Each of these samples exhibited a distinct and sharp band corresponding to an estimated size of approximately 550–600 base pairs, consistent with the expected amplicon size for fungi ITS regions (Khan & Bhadauria, 2019). The ITS region has become the obvious choice for the primary fungal DNA barcoding region due to increased accessibility of public databases. Moreover, it could analyse up to the genus and species level (Kauserud, 2023). The absence of any visible band in the negative control (lane marked "–") further validates the absence of contamination and supports the specificity of the PCR amplification in this study.

Notably, variations in band intensity are evident among the samples, with 11A62 and 11D2 producing visibly more intense bands compared to 11A61 and 11D3. These differences likely reflect variation in the efficiency of amplification, which may stem from differences in the concentration and quality of template DNA, as well as the degree of inhibitory substances remaining from the isolation process. Although A260/A230 ratios from Nanodrop spectrophotometry revealed suboptimal purity, as an indication of residual contaminants such as polysaccharides or chaotropic salts, the robustness of amplification suggests that these inhibitors did not reach concentrations sufficient to fully suppress Taq polymerase enzyme activity (Vajpayee *et al*., 2023). This is consistent with previous findings indicating that certain DNA polymerases exhibit partial tolerance to common PCR inhibitors (Manzo *et al*., 2024; Sidstedt *et al*., 2017).

The clarity and specificity of the amplicons indicate that the ITS primers effectively annealed to the target mold DNA and that the thermal cycling parameters used were appropriate for the amplification of degraded or partially impure DNA templates. Despite the presence of inhibitors suggested by spectrophotometric profiles, the presence of strong and specific bands across all samples reinforces the notion that the isolation protocol yielded DNA of sufficient functional quality for downstream molecular applications, particularly DNA barcoding and phylogenetic analysis.

These findings collectively underscore the importance of not relying solely on spectrophotometric purity indicators to assess DNA suitability for PCR. Rather, empirical validation through amplification remains essential, especially when working with complex samples such as mold samples that inherently contain high levels of inhibitory substances.

4. CONCLUSION

The results of this study confirm that Proteinase K effectively enhance the cell wall disruption in the lysis stage of silica column-based fungal DNA isolation. Its application led to increased genomic DNA yield and successfully amplified the Internal Transcribed Spacer (ITS) region. Therefore, the integration of Proteinase K could be recommended as an alternative additional step for commercial kits utilisation in fungal molecular research.

5. ACKNOWLEDGEMENTS

This study was funded by Riset Peningkatan Kompetensi (RPK) Universitas Jenderal Soedirman of the year 2023 to Y.E. and G.A.H. We thank other students of the microbiology study program, the Faculty of Biology for participating in this research.

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