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EXPEDITIOUS METHOD FOR GENOMIC DNA EXTRACTION FROM MUSHROOM MYCELIUM FOR DOWNSTREAM APPLICATIONS

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The immense diversity with morphological similarity in different mushrooms becomes a major problem for their identification which can be overcome by using molecular tools. Thus, rapid and efficient genomic DNA (gDNA) method from the mushroom mycelium is a key step to explore the molecular based research studies. The present study, standardized an efficient, quick and high throughput DNA extraction method from fresh fungal mycelium of different mushrooms. As compared with other available methods, this method requires less time and low quantity of mycelium (70-100 mg) to extract gDNA. It is time efficient and requires 2.0-2.5 hours for isolation of genomic DNA from single sample upto 16 samples in batch. This method excludes the thermal incubation for lysis and does not require lyophilization or use of liquid nitrogen. In this study, the results were validated using ITS (internal transcribed spacer) and SRAP (sequence-related amplified polymorphism) markers and compared with amplification obtained from DNA extracted from microwave method and CTAB method. In microwave method, in-complete or partial amplification was observed in SRAP marker.

However, microwave method was found to be suitable for internal transcriber spacer region amplification. In CTAB method, no amplification was found in SRAP marker. Our method showed an excellent amplification profile in both ITS and SRAP markers. gDNA extracted using this procedure holds good for molecular identification of mushroom species, diversity studies, DNA fingerprinting and downstream applications.

In the era of molecular studies, genomic DNA (gDNA) extraction is a key step in molecular research. DNA with high quantity and quality within quick time is important for many researchers. In mushrooms, the DNA is important for barcoding studies, diversity analysis, fingerprinting, etc (Kakoti *et al.*, 2021). Mushrooms are a diverse group of organisms and around 850 mushroom species are recorded from India (Ao *et al.*, 2016); out of which 283 are edible (Kaul *et al.*, 2019) . In mushrooms, look alike species create problems in taxonomic identification and hence also pose a threat of mushroom-poisoning, therefore, a high throughput protocol is needed to supplement the taxonomic studies for DNA extraction with applications in terms of identification at molecular level and diversity studies (Galliano *et al.*, 2021). Numerous gDNA extraction methods are reported in the literature for fungi and very few of them are consistent, inexpensive, reliable, arduous and quick. There are various methods for gDNA extraction in mushrooms as reported by Dentinger *et al.* (2010). The conventional cetyltrimethylammonium bromide- sodium dodecyl-sulfate (CTAB-SDS) method utilizes CTAB and SDS for disrupting the cell. This method is lengthy, needs a significant quantity of tissue, requires labour for gDNA extraction in multiple samples and loss of gDNA during the purification step (Moslem *et al.*, 2010). In general, there are three basic steps in DNA extraction i.e. lysis of cell, removal of protein and RNA and storage of DNA in buffers. Various researchers modify these steps to achieve success in gDNA extraction. For example, many researchers used liquid nitrogen for crushing the samples. Lyophilization is another approach to achieve easy crushing of samples for DNA extraction. Some researchers used dry ice for breaking the cells while some use enzymatic digestions and homogenization for DNA extractions (Griffin *et al.*, 2002). The microwave method was also used for lysing the cell for extracting DNA (Goodwin and Lee, 1993). Izumitsu *et al.* (2012) used microwave method in combination with cooling to isolate DNA in 16 minutes. Thermolysis is another technique for lysing the cell for extracting DNA (Zhang *et al.*, 2010). Oh *et al*. (2013) used ammonium sulphate for DNA extraction from mushrooms. The final DNA precipitation exploits many methods of purification such as silica binding technique (Karp *et al*., 1998; Katevatis *et al*.,2017), filter paper binding technique(Borman *et al.*, 2006; Shi *et al*., 2018), use of NaCl, glycogen (Bartram *et al*., 2009; Li *et al.*, 2020) and ethanol (Fregel *et al*., 2010). We also used such an approach to develop an efficient method for the extraction of gDNA from mushrooms.

The study was carried out in ICAR-Directorate of Mushroom Research (ICAR-DMR), on different mushroom species for the extraction of genomic DNA. The DNA extraction method was developed based on composition of buffers which was later compared with the traditional fungal SDS-DNA extraction and quick microwave method. The seven strains of mushroom

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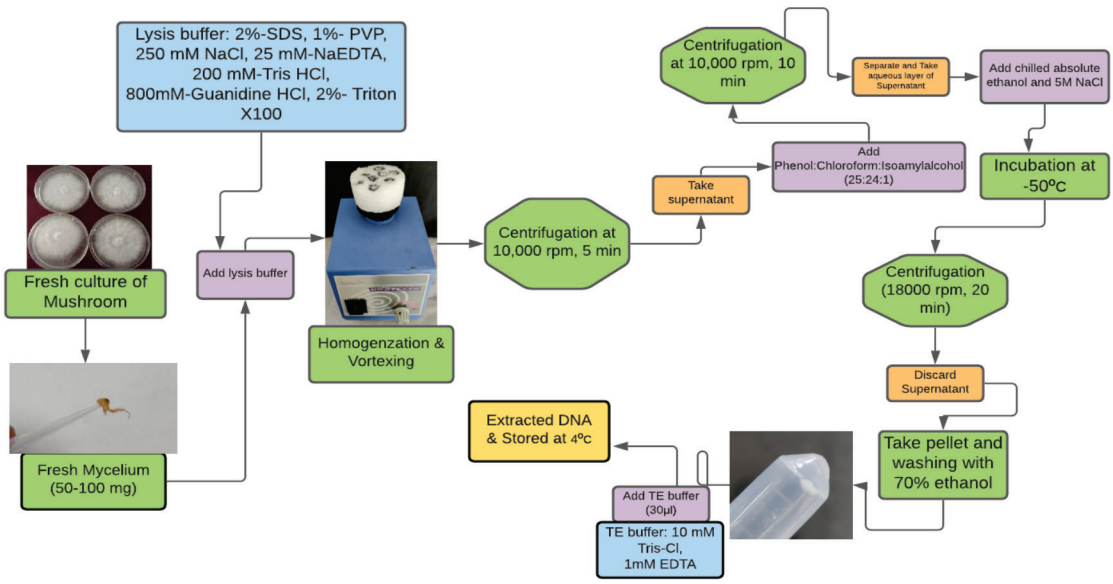
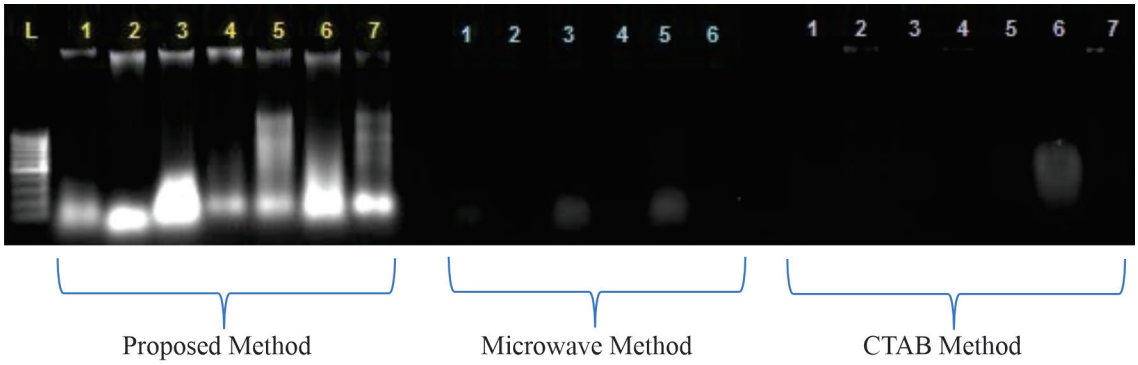


Fig. 1. Proposed DNA extraction method form mushroom mycelium

species were obtained from culture bank of ICAR- DMR culture bank, Solan *viz.*, *Agaricus bisporus* (NBS-5 and N-5-69), *Volvariella volvacea* (DMRO-463 and DMRO-484), *Lentinula edodes* (DMRO-702 and DMRO-23), *Calocybe indica* (DMRO-302 and DMRO-38).

The hyphal mycelium or mat mycelium weighing 70-100 mg was taken in an eppendorf tube. The 600 µl of lukewarm lysis (35-40°C) buffer {TRIS Buffer (tris(hydroxymethyl) aminomethane), Na_2EDTA (Ethylene diamine tetra acetic acid), Guainidine HCl, Triton x-100, SDS (Sodium Dodecyl Sulfate), PVP (polyvinyl pyrrolidone),Tween 20} was poured. The composition of lysis buffer is described in Table-2. The mycelia cell was lysed in Genei vortexer by making minor modifications (Fig.1). The vortex machine top was fitted with styrofoam with holes for placement of multiple eppendorf with samples. Genei bead of 7 mm was placed in each eppendorf and vortexed on scale of 6. The vortexing was done for 30 minutes. The vortexing process helps in disruption of the cells. After vortexing, the eppendorf was centrifuged at 10000 rpm for 5 min and supernatant was transferred to the new eppendorf. Protein removal was executed using Phenol-choloroform-isoamyl mixture (25:24:1) using an equal volume of obtained supernatant. The aqueous layer was transferred into new eppendorf and equal volume of ethyl alcohol was added, incubated for 30 minutes for -20 to -50°C. The solution was then centifiged at 4°C for 18000 rpm for 20 minutes. The obtained pellets were washed with 70% ethyl alcohol, air dried and disolved in 30-50µl TE (10mM Tris-Cl and 1mM EDTA) buffer. By using this method, DNA was extracted in 2.0 hours



Lane L*: 100 bp molecular marker;* Lane 1*: Agaricus bisporus* (NBS-5); Lane 2: *Calocybe indica* (DMRO-302); Lane 3: *Agaricus bisporus* (N-569); Lane 4: *Calocybe indica* (DMRO-38); Lane 5 and 6: *Volvariella volvacea* (DMRO-463 and DMRO-484) and Lane 7: *Lentinula edodes* (DMRO-702 and DMRO-23) for each extraction method.

Fig. 2. Comparison between proposed, microwave and CTAB method for extraction of genomic DNA

Table 1. Comparison between proposed method, microwave method and CTAB method for DNA quantity and purity value.

Strain	Proposed Method		Microwave method		CTAB method	
	DNA quantity (ng/µl)	Purity (=260/280 value)	DNA quantity (ng/µl)	Purity (=260/280 value)	DNA quantity (ng/µl)	Purity (=260/280 value)
Agaricus bisporus-(NBS-5)	401.0	1.89	256.5	1.53	46.5	1.28
Calocybe indica-DMRO-30	554.8	1.95	860.8	1.87	134.4	1.36
Agaricus bisporus-NBS-5-69	587.4	1.75	758.1	1.68	70.0	1.33
Calocybe indica- DMRO-38	386.6	1.81	133.9	1.61	57.4	1.79
Volvariella volvacea-DMRO-463	413.0	1.79	693.8	1.50	63.3	1.46
Volvariella volvacea- DMRO-484	467.5	1.73	484.0	1.33	113.5	1.24
Lentinula edodes- DMRO-702	446.0	1.71	495.0	1.59	167.8	1.01
Lentinula edodes - DMRO-23	455.0	1.78	690.0	1.55	42.7	1.31

compared to traditional method (Doyle and Doyle, 1987).

DNA concentration of samples was determined by Nano drop spectrophotometer and A260/A280 ratio were observed. The DNA was visually checked using gel electrophoresis by using 10 µl DNA in a 0.8% TAE-agarose gel, stained with ethidium bromide. The ITS and SRAP primers were used to carry out DNA amplification. The ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer was used for the amplification of a conserved internal transcribed region of the genomic DNA. For ITS amplification, DNA samples were amplified using Eppendorf thermo cycler with the following protocol. The initial denaturation was done at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s and extension at 72°C for 60s; a final extension phase was performed at 72°C during 10 minutes. Each reaction was carried out in 20 µl reaction volumes using 10 µl of Emerald amp Max PCR master mix (2X) reaction mixture, 0.4 µM of each primer, 7.2 µl nuclease free water and 2 µl template DNA. The electrophoresis of amplified DNA was carried in agarose gel (2.0% weight in volume, w/v) in 1 × Tris Acetate EDTA (TAE) buffer at 150 V for 2.5 hours,stained with ethidium bromide and then visualized under alpha-imager.

SRAP is a simple and efficient marker system that can be used in diversity studies, map construction, gene tagging, genomic and cDNA fingerprinting

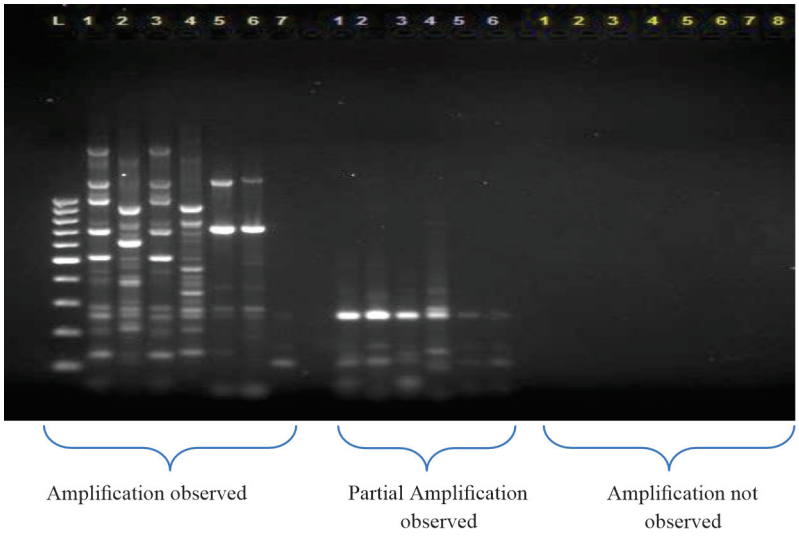


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Fig. 3. Amplification profile for SRAP markers (Me9Em2)

Table 2. Composition of Lysis Buffer used in study

Lysis buffer components	Concentrations	Role
TRIS Buffer (tris(hydroxymethyl) aminomethane)	200mM	Maintaining pH and osmolarity
Na_2EDTA (Ethylenediaminetetraacetic acid)	25mM	Deactivation of nuclease enzyme, metal chelator
Guainidine HCl	800mM	Denaturing proteins
Triton x-100	2%	Improves lysis strength
SDS (Sodium Dodecyl Sulfate)	2%	Disruption of cell membrane mainly protein part
PVP (polyvinylpyrrolidone)	1%	Helps to remove phenolic compounds
Tween 20	1 drop	Permeabilize cell membrane, solubilizing cell proteins

etc (Li and Quiros, 2001). Primers E9 (Forward-GACTGCGTACGAATTCAT) and M9 (Reverse-TGAGTCCAAACCGGAGG) was used in this study. The amplification was executed by initial denaturation at 94°C for 180 seconds, 5 cycles of denaturation (94°C for 60s), annealing (35°C for 90s) extension (72°C for 90s) again followed by 35 cycles of denaturation (94°C for 60s), annealing (50°C for 90s), extension (72°C for 90s) with final extension at 72°C during 8 minutes. Each reaction was carried out in 20 µl reaction volumes using 10 µl of Emerald amp Max PCR master mix (2X) reaction mixture, 0.4 µM of each primer, 7.2 µl nuclease free water and 2 µl template DNA. Amplified DNA was put to electrophoresis in agarose gel (1.5% weight in volume, w/v) in 0.5 × Tris-Borate EDTA (TBE) buffer at 150 V for 2.5 hours, stained with ethidium bromide and then visualized under alpha-imager.

Our method for extraction of genomic DNA of mushroom was found suitable for all mushrooms used in this study. The gDNA extracted from the method was found intact with minimal degradation (Fig. 2). The amount of gDNA varies from 200ng-600ng (Table-1). The DNA quantity was found significant for the primer amplification for identification and diversity analysis. Although we have not used RNAase treatment but can be used after homogenization and incubation at 37°C for 30 minutes. In our method, gDNA was easily isolated from either using mycelium or using fruit bodies. This method does not require lyophilization, but it can improve the DNA yield. The minimum amount of mycelium or tissue content is 70-100 mg fresh weight. The method has shown its suitability for PCR amplification using markers and also for molecular identification. In our study, ITS and SRAP amplification in comparison with microwave method and the CTAB method showed a good amplification result (Fig. 3). In microwave method, the DNA may be disrupted by microwaving, therefore, a complete amplification profile couldn't be achieved when compared with our method. However, microwave method was found suitable for ITS amplification. Therefore, microwave method may not be suitable for diversity of fingerprinting studies. In CTAB method, no amplification was observed in SRAP markers while good amplification was observed with ITS markers. In the past few years, many methods for gDNA extraction from mushroom mycelium have been performed but all these methods were tedious, time consuming and not reliable. The large differences in gDNA yields recovered may be due to variations in cell lysing and extraction method. We detected a significantly lower efficiency of extraction in CTAB method with lower mycelium quantity. The high quality of DNA extracted using the modified method was confirmed by the successful amplification of the ITS region using universal fungal specific primers.The microwave method alters cell walls and membranes, allowing lysis buffers to break cell and organelle membranes further. The methods can be used on mycelium and they are inexpensive because no expensive reagents or equipment are needed. The higher yielding DNA was observed but protein contamination was seen in many fungal mycelial strains, that contamination further leads to degrading of DNA with long term preservation and partial amplification was observed using SRAP molecular markers (De *et al.*, 2007). Therefore, we cannot observe the genetic variation within diversified mushroom from microwave method.

The present method also avoids the step of developing fruitbodies for large number of fungal specimens or cultures. The current gDNA extraction is useful for molecular breeding and gene studies. This proposed DNA extraction method employs bead and vortex equipment to effortlessly lyse many mushroom mycelium in a short period of time. Our findings indicated that the overall quality and yield of gDNA is influenced by proposing DNA extraction approach. The gDNA quality is significantly higher as compared to other extraction methods. This method proved to be the best because it is simple, less time consuming, inexpensive, consistent (yield) and reliable than other methods. Mushroom mycelial strains were also amenable to PCR amplification and this method can be adapted on routine basis for downstream applications *viz*. genomic, metagenomic and transcriptomic study.

Authors' contribution

Conceptualization of research work and designing of experiments (AB); Execution of field/lab experiments and data collection (KS, AB); Analysis of data and interpretation (MN, SK); Preparation of manuscript (VPS)

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