CHAPTER III

MATERIALS AND METHODS

MATERIALS

A. Mycelia material

The mycelia used in the study were shiitake mushroom isolates or *Lentinus edodes* (Berk.) Sing strains MuL2, MuL4, MuL5, MuL9/2, MuL9/4, MuL11 and MuL12 which were kindly supplied by Associate Professor Mukda Kuhirun, Mushroom Research Unit, Department of Botany, Chulalongkorn University and Japanese cultivar (Appendix I-5). The mycelia used were in form of the secondary mycelia (n+n) on Potato Dextrose Agar (PDA) slants.

B. Molecular weight standard markers and primers.

- UBC primers (University of British Columbia Biotechnology Laboratory) UBC101, UBC174, UBC228, UBC268, UBC273, UBC299, UBC428, UBC456, UBC457 and UBC459 were supplied by Dr. Sirawut Klinbunga, Dapartment of Marine Science, Chulalongkorn University (Appendix I-6).

- 100 base pair (bp) DNA ladder (15 fragments: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400 and 1500 bp) concentration 250 μg/ml. In storage buffer = 1μg/μl in 10 mM Tris-HCl; 1mM EDTA, pH 8.0

C. Instruments

- Autoclave, model TM-328, Tomin Medical Equipment Co., Ltd.
- Automatic micropipette P10, P20, P200, P100 (Gilson)
- Electronic balance with 2 digits, model U4600 P, Sartorius, Germany
- Electronic balance with 4 digits, model 80A-200M, Presica, Switzerland
- Homogenizer, The Virtis Company, Inc., Gardiner, New York, USA.
- Incubator, model UM100, Memmert Gmbh Co., Germany
- Microcentrifuge, Mikro 12-24, Hettich Zentrifugen, Germany.
- PCR, Hybrid OmniGene, HBTR3CM, Hybaid Ltd., UK.
- Power supply, Power PAC 300, Bio-Rad Laboratories, USA.
- Vertical gel electrophoresis apparatus (16X16 cm), ATTO Corporation, Japan

D. Inventory Supplies

- Color print film, Gold 200, Eastman Kodak Company Rochester, USA.
- Conical tube 15 ml, Nunc., Germany
- Cuvette 1 ml Quant. 4 Type 8 Q 10 mm., Strana Ltd.
- Eppendorf centrifuge tube 0.5 ml, Axygen Hayward, USA.
- Eppendorf centrifuge tube 1.5 ml, Treff AG, CH-9113 Degersheim, Switzerland.
- Erlenmeyer flasks 250 ml, Pyrex, USA.
- Fillter paper 90 mm ∅ No. 4 Whatman International Limited, England.
- Glass pasteur pipette X 225 mm, Brand Gmbh Co., W. Germany.
- Glass vacuum filter holder, Sartorius AG, Goettingen, Germany.
- Parafilm 4 in. x 125 ft, Parafilm M laboratory film, Chicago, USA.
- Pipette tips 10, 20, 100 μl, Treff AG, CH-9113 Degersheim, Switzerland.
- Pipette tips 1000 μl, Rainin Instrument Co., USA.
- Spectrophotometer, GENESYS 5, Milton Roy Company, USA.
- Syringe 50 ml, Terumo Corporation, Tokyo, Japan
- Water bath, model SU5, Grant Instruments (Cambridge) Ltd. Barrington, England

E. Chemicals

- Acetic acid glacial (CH_3COOH 100%, MW. 60.05 g/mol), Merck, Germany.
- Acrylamide ($CH_2=CHCONH_2$), USB, American International plc., England.
- Ammonium persulfate ($(NH_4)_2S_2O_8$), USB, American International plc., England.
- Bacto-agar, Difco Laboratories, Detroit, Michigan, USA.
- Bacto-malt extract, Difco Laboratories, Detroit, Michigan, USA.
- N, N'- Methylene-Bis-Acrylamide ($(CH_2=CHCONH)_2CH_2$), USB, American International plc., England.
- Boric acid (H_3BO_3, MW 61.38 g/mol), Farmitalia Carbo Erba, Milano, Italy.
- Bromophanol blue, Merck, Germany
- Cetyl trimethyl ammonium bromide (CTAB, MW 364.46 g/mol), Merck, Germany
- Chloroform ($CHCl_3$, MW 119.38 g/mol), Merck, Germany
- Citric acid monohydrate ($C_6H_8O_7.H_2O$, F.W. 210.14), J.T. Baker Chemicals B.V., Deventer, Holland
- Disodium ethylenediametetraacetic acid-2-hydrate ($Na_2EDTA.H_2O$, MW 372.24 g/mol), Fluka Chemica, Biochemica, Switzerland
- Ethanol absolute (C_2H_5OH, MW 46.07 g/mol), Merck, Germany
- Ethylene diamine tetra-acetic acid (EDTA), Fluka chemica, Biochemica, Switzerland.
- Formaldehyde 40% m/v (HCHO, M 30.026), Farmitalia Carlo Erba, Milano, Italy.
- Formamide ($HCONH_2$, M 45.04 g/mol), Merck, Germany.
- Gene Amp. PCR core reagent (Perkin Elmer Cetus, USA.)
  : 10 x Taq DNA polymerase storage buffer (100 mM Tris-HCl, pH 9.0 at 25 °C, 500 mM KCl, 1% Triton X-100, Magnesium-free), Promega Co., USA.
  : 10 mM dNTPs nucleotide mixes (dATP, dCTP, dGTP, dTTP), Promega Co., USA.
  :Taq DNA polymerase (5 U/μl), Promega Co., USA.
  : Magnesium chloride (25 mM), Promega Co., USA.
- Glycerol ($CH_2OH.CHOH.CH_2OH$, MW 92.10 g/mol), BDH Chemicals Ltd., England.

- Glucose monohydrate ($C_6H_{12}O_6.H_2O$, MW 198.17 g/mol), Merck, Germany.

- Hydrochloric acid (HCl 37%, MW 36.46 g/mol, May and Baker Laboratory Chemicals, England.

- 8 - Hydroxy-quinoline (8-hydroxy-1-azanopthalene, C_9H_7NO, FW 145.2), Sigma Chemical Company, USA.

- Iso-amyl alcohol ($C_5H_{11}OH$, MW 88.151 g/mol), Merck, Germany.

- 2-Mercaptharol (2-hydroxyethylmercaptan; β- Mercaptoethanol, C_2H_6OS, FW 78.13), Sigma Chemical Company, USA.

- Mineral oil, Aldrich Chemical Company, Inc., Germany.

- Methyl alcohol (CH_3OH, M 32.042), Farmitalia Carlo Erba, Milano, Italy.

- Nitric acid (HNO_3, 70%), Farmitalia Carlo Erba, Milano, Italy.

- Phenol crystal (C_6H_5OH), Merck, Germany.

- Potato dextrose agar, Difco Laboratories, Detroit Michigan, USA.

- Potato dextrose broth, Difco Laboratories, Detroit Michigan, USA.

- Polyvinyl pyrrolidone (PVP type 360), Sigma Chemica Company, USA.

- Proteinase K, Merck, Germany.

- Silver nitrate ($AgNO_3$, MW 169.87 g/mol), BDH Laboratory, England.

- Sodium acetate (CH_3COONa, FW 136.08), Farmitalia Carlo Erba, Milano, Italy.

- Sodium carbonate anhydrous (Na_2CO_3, MW 105.99 g/mol, Fluka Chemica Biochemica, Switzerland.

- Sodium chloride (NaCl, MW 58.44 g/mol), BDH Chemicals Ltd., England.

- Sodium dodecyl sulfate (SDS, FW 288.38), USB, United States Biochemical.

- Sodium hydroxide pellet (NaOH, MW 40 g/mol), Merck, Germany.

- Tris - (hydroxymethyl – aminomethane ($C_4H_{11}NO_3$, FW 121.1), Serva, Feinbiochemica GmbH & Co., France.

- TEMED ($C_6CH_{16}N_2$, FW 116.21), USB American International Co., England.

- Urea (CH_4N_2O, MW 60.06 g/mol), Merck, Germany.

- Xylene Cyanol FF, Sigma Chemical Company, USA.

- Yeast extract, Difco Laboratories, Detroit Michigan, USA.

METHODS

A. Cultivation of mycelia from 8 isolates of *Lentinus edodes*

To prepare a suitable study method for DNA extraction and find the appropriate condition as a simple method for mycelia cultivated, several isolates were used to test for the variable of culture media. The steps test were devided as follows.

1. Culturing and harvesting of shiitake mushroom isolates

Eight isolates of the shiitake mushroom were cultured by placing the preserved mycelia from stock culture on PDA, after colonization, the obtained mycelia were sworbed, transferred to 50 ml of PDB, and incubated in dark condition at 25 °C until needed.

2. Studies on a suitable liquid culture of *L. edodes* for DNA preparation.

In order to maximize the amount of DNA during preparation, good quality of starting mycelia were used. In this study, the suitable liquid culture mycelia was suitable to provide rapid young mycelia cultured.

The inoculum was prepared after 10 days in incubation on PDB and tested to determine the best condition suitable for mycelial DNA preparation from 8 isolates on PDB. This was done by the homogenization of fresh mycelia and inoculation on 150 ml of the different liquid culture formulations (Appendix I-7) and incubated with a slow shaking at 25 °C for 20 days. The mycelia from each condition were collected every 5 days and data on fresh weight was collected after pelleting the mycelium in eppendorf tubes. The mycelia were then collected on Whatman filter paper No.1 and dry weight was measured after incubation at 60 °C for 24 hrs. Mycelia growth in each liquid culture formulations and MYG liquid medium was compared by the procedure described in Fukuda *et al.* (1994).

This experiment was conducted in complete Split Plot Design with four replications and the data on the mycelia growth of *L. edodes* was statistically analyzed.

The result were used as a reference to determine the best liquid culture condition for rapid mycelium cultivation which provided good starting material for DNA extraction.

B. Development of a simple and rapid method for the preparation of *Lentinus edodes* genomic DNA for RAPD analysis.

In order to develop a simple and rapid DNA extraction method suitable for the DNA analysis, the main criteria was to complete the extraction within a day without handling any hazardous organic solvents. This should also be appropriate for DNA preparation from many samples simultaneously, this would reduce steps of extraction that included the removal of polysaccharides that protect the DNA from the activity of endonuclease. The method was based on a rapid procedure that used only milligram quantities of mycelia and could provide sufficient amount of DNA for further DNA analysis.

Total genomic DNA was isolated from the mycelia using Standard method as described by Sambrook *et al.* (1989). Approximately 100 mg of 15-day-old mycelia were added to 500 μl phenol and 200 μl extraction buffer and then ground to fine powder. The solution was transferred into an eppendorf tube. The tube was vigorously shaken before being centrifuged at rpm_{max} for 3 min. The supernatant was transferred to a new eppendorf tube. Sn equal volume of phenolchloroform was added, followed by a vigorous shake before being centrifuged at rpm_{max} for 3 min. An equal volume of Isoamylchloroform was added to the supernatant and the tube was shaken again before being centrifuged at rpm_{max} for 3 min. One-tenth volume of Sodium acetate pH 5.2 was added and mixed well, followed by an addition of 2.5 volumes of absolute ethanol. The solution was mixed by gently inverting the tube several times, then stored at -20°C for 2 hr until the DNA precipitate appeared. The solution was

centrifuged at rpm_{max} for 15 min and the precipitated DNA pellet was washed by 70% ethanol (v/v), then, the ethanol was removed as much as possible by slightly inverting the eppendorf tube carefully. The DNA pellet was air-dried for 1 hr.

The Glass bead method was described in Waiblinger *et al.* (1999). Approximately 100 mg of 15-day-old mycelia was extracted using 100 mg of Glass bead in Eppendorf tubes, mixed by vortex for 1 min. 800 μl of 1% CTAB precipitation buffer was added and incubated at 65°C in a water bath, and shaken for 1 hr. 20 μl of Proteinase K solution (20 mg/ml) was added to each tube, and incubated at 55°C for 1 hr. The suspension was centrifuged at rpm_{max} for 10 min. The clear lysates were extracted with phenolchloroform v/v, followed by ethanol precipitation (Sambrook *et al.*,1989).

The SDS extraction method was described in Lee and Taylor (1991). Approximately 100 mg of 15-day-old mycelia was mixed with 400 μl lysis extraction buffer then ground to fine powder. The solution was incubated at 65 °C for 1 hr. 400 μl phenolchloroform was added vigorously shaken before being centrifuged at rpm_{max} for 15 min. The supernatant was added 1/3 volume of 3M NaOAc and 0.54 volume of Isopropanol. The solution was mixed by gently inverting the tube several times until the DNA precipitate appeared. The DNA precipitate was centrifuged at rpm_{max} for 2 min. The DNA pellet was washed with 70% ethanol and air-dried.

The Urea extraction method was described in Cherdshewasart (1991). Approximately 100 mg of 15-day-old mycelia was added to 500 μl urea extraction buffer and 500 μl phenolchloroform then ground to fine powder. The solution was transferred into a new eppendorf tube and stored at room temperature for 15 min. The tube was centrifuged at rpm_{max} for 10 min. One-tenth volume of NaOAc was added to the supernatant (Sambrook *et al.*, 1989).

The Chelex extraction method was described in Walsh, Metzger and Higuchi (1991). Approximately 100 mg of 15-day-old mycelia were added to 200 μl chelex and

1000 μl TE then ground to fine powder. The solution was transferred into a new eppendorf tube and boiled for 8 min. The tube was vortex for 5-10 sec and then centrifuged at rpm_{max} for 3 min. The supernatant was extracted again with an equal volume of phenolchloroform (Sambrook *et al.*, 1989).

The CTAB extraction method followed the procedure described in Sambrook *et al.* (1989). Frozen mycelia were ground to fine powder. The powder was transferred to a new eppendorf tube containing 2xCTAB and incubated at 55°C, for 10 min, then an equal volume of isoamylchloroform was added. The solution was mixed by shaking for 30 min before being centrifuged at 2800 rpm for 15 min. The pellet was added 1xCTAB for 10 min before another round of centrifugation. The supernatant was added an equal volume of isoamylchloroform and centrifuged at 2800 rpm for 10 min. One-tenth volume of 10% CTAB was added and mixed by slightly converting, then 1 volume of 1% CTAB was added followed by incubation at room temperature for 30 min. The solution was centrifuged at 2800 rpm for 15 min. The pellet was dissolved in 500μl of NaCl-TE. The DNA was precipitated in an equal volume of isopropanol before being centrifugated 2800 rpm for 10 min. The pellet was washed by propanol and air-dried.

The treatment of NaOH was described in Sambrook *et al.* (1989). Approximately 100 mg of 15-day-old mycelia was mixed with 0.5N NaOH. The solution was incubated at 65°C for 10-15 min. Five μl of the solution was added to 495 μl of 0.1M Tris, pH 8.0 and was stored prior to use at -20°C.

The DNA pellets were redissolved in 20 μl sterile DW. DNA concentration was measured with a spectrophotometer at 260, 280, and 320 nm. The ratio of $A_{260/280}$ also calculated. The concentration of DNA samples were adjusted to 10 ng/μl for further use in DNA analysis and stored at -20°C.

C. RAPD analysis of the genomic DNA

In this study, PCR-based technique of RAPD or DAF was applied to distinguish the variation in the genomic DNA of the shiitake mushroom isolates. The detailed steps were as follow.

1. PCR amplification

To test the random primers, the 10-decamers (Appendix I-6) were chosen randomly and used for genomic DNA-PCR amplification (Fritsch *et al.*, 1993). These primers were supplied by Dr. Sirawut Klinbunga, Department of Marine Science, Faculty of Science, Chulalongkorn University.

Amplification reactions were carried out in a 20-microliter reaction volume containing 10x buffer (Promega Co., USA), 2 mM $MgCl_2$, 0.2 mM of each dNTP nucleotide mixes, 0.2 μM Primer (University of British Columbia Biotechnology Laboratory), 2U Taq DNA polymerase and 25 ng DNA template. The reactions were overlaid with mineral oil and amplified in a thermocycler (Hybaid Omni Gene, U.K.) with a condition of 35 cycles, a denaturation at 94 °C for 15 sec., an annealing at 36 °C for 45 sec, and an extension at 72°C for 90 sec. The resulted products were kept for further analysis.

2. PAGE and silver staining

The fragment lengths of the amplified DNA were determined on 8% polyacrylamide gel electrophoresis on basis of their molecular size. The DNA mixture consisted of 2 μl 10xTBE loading buffer (Appendix IV), 16 μl formamide, 1 μl sterile DW and 1 μl amplified DNA. The DNA fragment lengths were determined against the known 100-base-pair ladder (Boehringer Mannheim, Singapore). Polyacrylamide gel electrophoresis was performed with 0.5xTBE as running buffer, at a constant voltage of 120 V for 15 hr. The gels were stained with silver stain technique as described in

Berry and Samuel (1982), and DeMoreno, Smith, and Smith (1985). The gels were then cast to dry for long term storage by transferring onto a wet cellophane sheet and drying the gels at room temperature. The gels were photographed for permanent record. All the amplifications were tested at least twice for reproducibility.

D. Data Analysis

Photographs from silver stained polyacrylamide gels were used for RAPD analysis. DNA fragment data were scored for computer analysis based on the presence or absence of the amplified products. If a product was present, it was designated "1", if absent, it was designated "0". Pair-wise comparisons of these studies were analyzed with the computer software for Jaccard's similarity coefficient (Jaccard, 1908). The obtained similarity coefficients were then used to construct a dendrogram using Unweighted Pair-Group Method with Arithmatical Average (UPGMA method, version 3.572c).

The resulting dendrogram and distance provided the estimates of genetic distance and relationships among isolates of shiitake mushroom.