

CHAPTER IV

RESULTS

A. Studies on a suitable liquid culture condition for maximum mycelial growth rate of *Lentinus edodes* for DNA extraction.

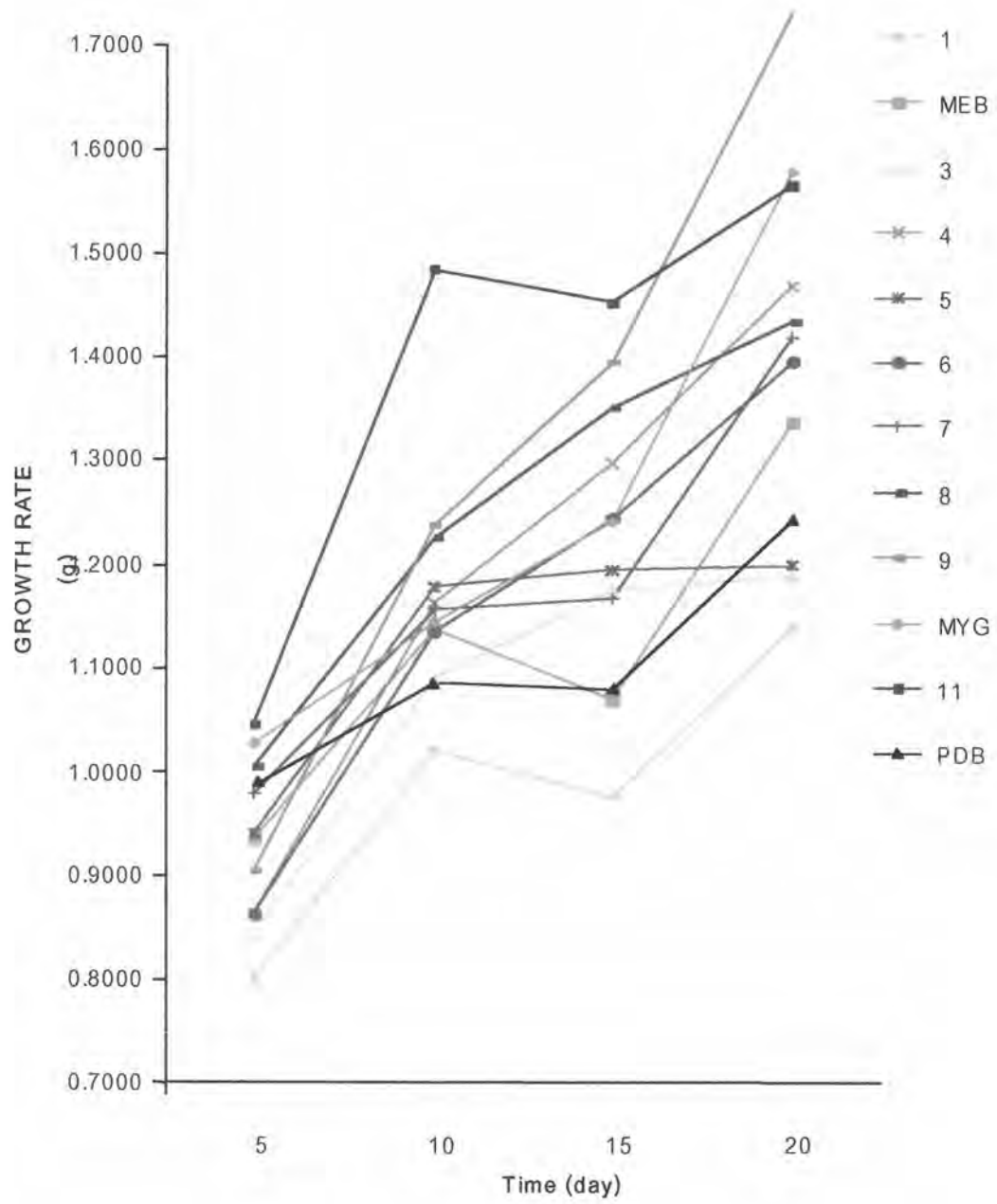
The cultivation system could be developed to provide suitable early mycelial growth for DNA extraction with criteria on rapidity and simplicity as priority consideration. Mycelia of *L. edodes* were cultivated in 12 different liquid media formulations including the recently published MYG liquid medium by Fukuda *et al.* (1989), MEB, PDB and liquid culture formulations 1, 3-9 and 11.

Measurement of mycelial dry weight from different culture conditions collected in the period of 5 days showed that mycelial growth rate from 12 conditions was not significantly different, however, when growth rate in terms of cumulative dry weight was further observed in the following 5 or 10 days, the best mycelial growth rate was observed with the liquid culture formulation 11 (Fig. 1), followed by formulations 9, 7, and MYG liquid media. During this second period of 5 to 10 days mycelia grew rapidly resulting in a greater amount at dry weight accumulation, and the proliferation then decreased slowly and constantly on the day of 20 in all formulations. A similar trend was also observed in fresh weight accumulation (Fig. 2).

With reference to the plotted curve at 15 days, the mycelial dry weight obtained from liquid medium formulation 11 was 0.6593 g whereas that of others varied from 0.5726 to 0.6489 g, respectively (Appendix II-1).

These results suggested that liquid culture formulation 11 can provided a condition to induce better mycelial growth in *L. edodes* within a short period of time.

Figure 2 : The mycelial growth rate in fresh weight of *Lentinus edodes* cultured on different liquid culture formulations



By using this formulation, it was possible to promote growth activity and cell enlargement that would be sufficient for DNA preparation. Therefore, this formulations was suitable for cultures in order to prepare mycelia for DNA extraction and RAPD analysis in further step.

B. Comparative studies on methods for *Lentinus edodes* genomic DNA extraction for PCR analysis.

There had been an attempt to define a suitable method for *L. edodes* DNA extraction. There were several methods used in the preparation of DNA from plants, fungi, and animal cells which had been applied to extract DNA from mycelia prepared in the previous steps.

The DNA quality as well as quantity was checked. Results showed that the majority of DNA molecules varied from high to low molecular weight (Fig. 3) with a standard method. The obtained DNA appeared as dark smear bands reflecting the amount of DNA yield in equal nanogram amount of mycelia used. In the studies neither the CTAB method nor the NaOH method nor the Chelex method gave good result.

Good DNA quality was judged by visualizing the gel after silver staining. Relatively similar DNA products were obtained with SDS method, Glass bead method, Standard method and Urea extraction method.

The quality of DNA in terms of their purity and quality of amplification was determined as OD ratio at 260:280 and OD 320; and the amount was quantity as DNA yield. Results in Table 1 showed that among the methods used, Glass bead method gave a similar efficiency to those of the standard at the second efficiency level, while the SDS assay and Urea extraction did not show a different result.

Standard method gave a high quality DNA (OD_{260:280} ratio of 1.7) and gave the higher yield of DNA (3.38 $\mu\text{g}/\mu\text{l}$) than other methods tested.

Figure 3 : *Lentinus edodes* DNA prepared from different techniques after electrophoresed on a 8% PAGE for 7 hr at 120 V at room temperature. Lanes 1 and 9 contained a 100 bp DNA ladder marker. Lanes 2, 3, 4 showed the very small amounts of DNA which were extracted by using CTAB, NaOH and Chelex. Lanes 5, 6, 7 and 8 showed very large amounts of DNA by using SDS, Glass bead, Standard (phenol) and Urea methods, respectively.

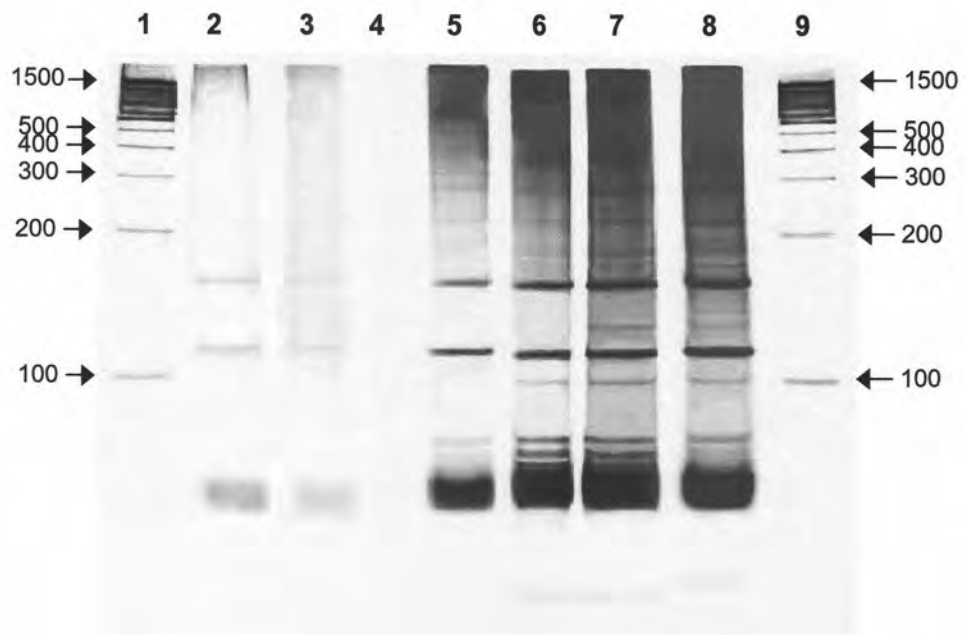


Table 1 : Average quantity of DNA from different extraction methods using 100 mg mycelia from 5 replications.

DNA extraction method	OD₂₆₀ : OD₂₈₀	OD₃₂₀	DNA yield (μg/μl)
Standard	1.74	0.014	3.38
SDS lysis	1.76	0.008	2.04
Urea extraction	2.31	0.012	2.08
CTAB extraction	1.76	0.006	1.80
5% Chelex	2.00	0.003	0.12
NaOH	1.13	0.004	0.34
Glass bead	1.70	0.006	2.92

C. RAPD analysis

1. Amplification of *Lentinus edodes* DNA

Ten different arbitrary oligonucleotide decamers were tested as primers to amplify DNA segments from the genomic DNA from 8 strains of *L. edodes*. No amplification product was observed with primers UBC101, UBC174, UBC268, UBC273, UBC299 and UBC428. Successful amplifications of DNAs from *L. edodes* isolates were observed with primers UBC228 (GCTGGGCCGA), UBC456 (GCGGAGGTCC), UBC457 (CGACGCCCTG) and UBC459 (GCGTCGAGGG) which were resolved by using 8% polyacrylamide gel electrophoresis and silver staining (Fig. 4, a-d). The figure showed DNA fragments with size range from 150 to 1500 bp of the primers tested. UBC456 gave the greatest number of amplified fragments. All amplification was repeated two to seven times. However, variation was observed in the relative amounts of certain DNA segments.

Amplified polymorphic DNA fragments were then scored as described in Materials and Methods for computer analysis.

2. Genetic similarity among isolates

Of all the 101 amplified products, 51(50.5%) of MuL2, 56(55.45%) of MuL4, 62(61.39%) of MuL5, 49(48.52%) of MuL9/2, 52 (51.49%) of MuL9/4, 55 (54.46%) of MuL11, 54(53.47%) of MuL12 and 53(52.48%) of the Japanese cultivar were polymorphic (Table 2). For each primer evaluated, the number of amplified products ranged from 22 to 28. Amplified polymorphic DNA fragments were scored for computer analysis based on Jaccard's similarity coefficients and ranged from 0.193548 to 0.646341 (Table 3). The table showed genetic distance of 8 *L. edodes* isolates indicated that MuL12 was the most distantly related to other isolates. Association among the 8 strains of *L. edodes* were divided into two main clusters, A and B, on the basis of Jaccard's similarity coefficients from the results obtained with UBC228, UBC456, UBC457 and UBC459 primers (Fig. 5).

Table 2 : Number and percentage of DNA polymorphisms when synthetic arbitrary oligonucleotides were used as primers for RAPD analysis in 8 *L. edodes* isolates.

Primer number	Nucleotide sequence (5' to 3')	No. of amplified fragments (a)	Isolates of <i>L. edodes</i>	No. of polymorphic fragments (b)	% polymorphic (b/a x 100)
UBC228	GCTGGGCCGA	26	MuL2	12	46.15
			MuL4	20	76.92
			MuL5	19	73.08
			MuL9/2	17	65.39
			MuL9/4	20	76.92
			MuL11	20	76.92
			MuL12	18	69.23
			Japan	13	50.00
UBC458	GCGGAGGTCC	28	MuL2	16	57.14
			MuL4	13	46.43
			MuL5	15	53.57
			MuL9/2	14	50.00
			MuL9/4	13	46.43
			MuL11	23	82.14
			MuL12	12	42.86
			Japan	16	57.14
UBC457	CGACGCCCTG	22	MuL2	7	31.82
			MuL4	8	36.36
			MuL5	10	45.46
			MuL9/2	9	40.91
			MuL9/4	9	40.91
			MuL11	3	13.64
			MuL12	13	59.10
			Japan	6	27.27
UBC459	GCGTCGAGGG	25	MuL2	16	64.00
			MuL4	15	60.00
			MuL5	18	72.00
			MuL9/2	9	36.00
			MuL9/4	10	40.00
			MuL11	9	36.00
			MuL12	11	44.00
			Japan	18	72.00
	TOTAL	101	MuL2	51	50.50
			MuL4	56	55.45
			MuL5	62	61.39
			MuL9/2	49	48.52
			MuL9/4	52	51.49
			MuL11	55	54.46
			MuL12	54	53.47
			Japan	53	52.48

Table 3 : Genetic distances of 8 *Lentinus edodes* isolates based on Jaccard's similarity coefficients.

Isolates *	Japan	MuL12	MuL11	MuL9/4	MuL9/2	MuL5	MuL4	MuL2
Japan	-							
MuL12	0.64634	-						
MuL11	0.62195	0.44444	-					
MuL9/4	0.31818	0.64198	0.37313	-				
MuL9/2	0.25000	0.38806	0.64557	0.31746	-			
MuL5	0.40278	0.38356	0.42857	0.62069	0.41333	-		
MuL4	0.35135	0.34849	0.19355	0.38028	0.59259	0.31313	-	
MuL2	0.29230	0.46053	0.40000	0.32813	0.38235	0.62025	0.28571	-

* The following abbreviations are used for the isolates of *Lentinus edodes* as indicated in Appendix I-4.

Cluster A was subdivided into four subclusters (A1, A2, A3 and A4): subcluster A1 were isolates from Japan (MuL4, MuL9/2 and MuL 9/4); subcluster A2 were isolates from Taiwan (MuL2) and Japanese cultivar; subcluster A3 was from an Taiwan cultivar (MuL11); and subcluster A4 was an isolate from Japan (MuL5). Cluster B consisted of MuL12, a the wild cultivar of Thailand.

The difference between clusters A and B were the most evident when the UBC456 and UBC459 primers were used (Fig. 4b and d). The results of comparison indicated that MuL12 was the most distantly related to all the other isolates of *L. edodes*. Within subcluster A1, there was indication that this group was more genetically homogeneous than A2 and A3, and subcluster A2 was more genetically homogeneous than A3 and A4.

Figure 4 : Patterns of Random Amplified Polymorphic DNA detected in 25 ng of *Lentinus edodes* (Berk.) Sing genomic DNA on an 8% polyacrylamide gels and silver staining. Lanes 1 and 10 contain 100-bp DNA ladder marker, lanes 2-9 were MuL2, MuL4, MuL5, MuL9/2, MuL9/4, MuL11, MuL12, Japanese cultivar. The primers were UBC228 (a), UBC456 (b), UBC457 (c) and UBC459 (d).

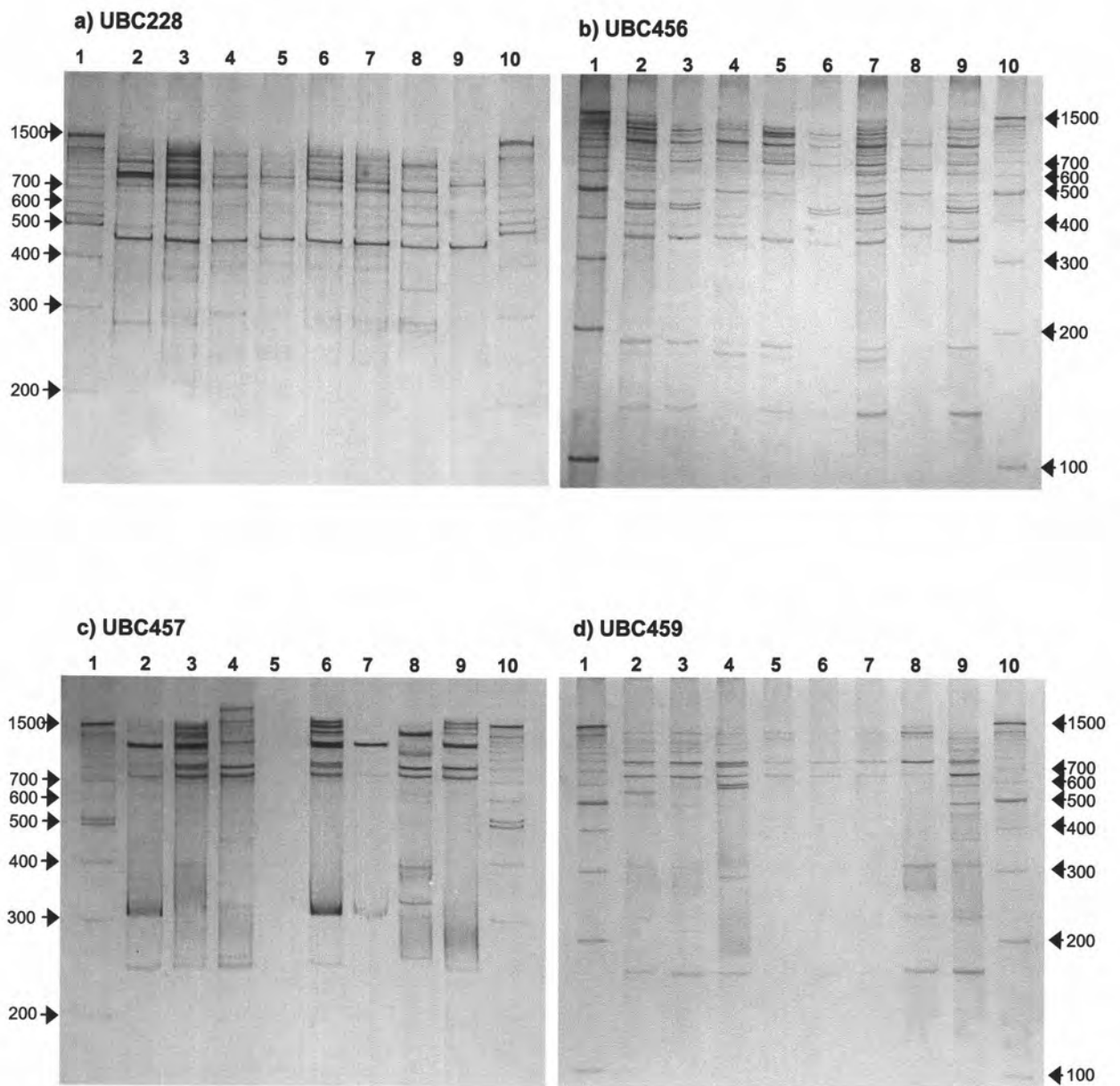


Fig 5 : A UPGMA dendrogram based on Jaccard's similarity coefficients illustrating the genetic relationships among 8 *Lentinus edodes* isolates. Relative lengths indicate similarity indices. The abbreviations of samples are the same as those indicated in Appendix I-5.

