SYMMETRICAL RECOVERY OF MONOKARYOTIC COMPONENTS FROM *LENTINULA EDODES* USING DEDIKARYOTIZATION

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ABSTRACT

The dedikaryotization method proposed for *Pleurotus* spp and other basidiomycetes by Leal-Lara and Eger-Hummel in 1982 was modified in order to recover the monokaryotic components of seven *Lentinula edodes* strains. By decreasing the time that agar cultures were blended, mycelial death was reduced and more neohaplonts were recovered. If agar culture homogenates were plated on malt extract agar, *L. edodes* strains barely survived 30 seconds blending. Agar cultures of *L. edodes* blended for more than 5 seconds did not yield viable mycelium when agar culture homogenates were inoculated in dedikaryotization liquid media. Optimum blending time of agar cultures was determined for each strain. An attempt was made to recover neohaplont types at different stages of the dedikaryotization procedure either directly from agar culture homogenates or from dedikaryotization media inoculated with such homogenates and incubated for 14 days at 24ºC or from homogenates incubated for fourteen days in dedikaryotization media. All seven *L. edodes* strains were successfully dedikaryotized. In all cases, both monokaryotic components were recovered and in six cases, they were isolated in a 1:1 ratio.

INTRODUCTION

Lentinula edodes is an edible fungus which is not easy to cultivate, because it is grown in substrates difficult to degrade and long incubation periods are required. Therefore, selection of *L. edodes* strains able to grow rapidly on a wide range of substrates and

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produce high yields is of utmost importance. However, breeding of improved strains by conventional methods is a labor intensive and time consuming procedure (Eichlerová and Homolka 1999). Dedikaryotization represents an interesting alternative, since it allows recovery of the two monokaryotic components (also known as neohaplonts) of selected dikaryotic strains. By pairing neohaplonts from selected stocks, new strains can be easily produced, in which the desired characteristics of the original stocks are combined. The deleterious side effects which are frequently produced by mutation are thus avoided. This straight-forward method shows potential for producing commercial strains with defined characteristics, and may be superior to the conventional breeding method, which randomly pairs monokaryons from meiotic offsprings.

Dedikaryotization was initially attempted with *Schizophyllum commune* (Harder 1927) using a micro-surgery approach for a mechanical separation of the two nuclei of a terminal cell. Two cells, each one containing one nucleus, were recovered by cutting the clamp cell from the terminal cell, at the moment when one nucleus had migrated into the clamp connection. However, environmental factors, such as temperature, humidity and type of nutrients, favored a low recovery of neohaplonts (20%). Fries and Aschan (1952) confirmed that *Pholiota mutabilis*, *S. commune*, *Polyporus abietinus* and *Collybia velutipes* neohaplonts were difficult to recover by this procedure. Ginterová (1973) suggested a second approach for mechanical dedikaryotization – the application of shearing forces during homogenization at high speed. However, dedikaryotization was again unsymmetrical, since the most sensitive nucleus was selectively eliminated and only one nucleus was recovered.

Milles and Raper (1956) introduced chemical dedikaryotization with toxic substances producing selective inactivation of one nuclear type, and causing asymmetrical splitting of the dikaryon. Using sodium taurocholate (0.15%) or colic acid (0.12%) success was attained with stocks which produced monokaryotic oidia or with strains of *Favolus alveolaris*, *Pleurotus ostreatus*, *P. betulinus* and *P. versicolor* . However, these already showed a high level of natural dedikaryotization (60%). Other derivatives of cholic acid, such as sodium cholate or ox-gall produced dedikaryotization of *C. velutipes*, *Coprinus macrorhizus*, *L. edodes* and *Pholiota nameko* (Takemaru 1964). However, each species required a specific concentration for its dedikaryotization and only one nuclear type was recovered in all cases. Additionally, morphological mutants were frequently observed and the serious inhibition of mycelium growth produced by toxicants, resulted in long incubation periods to achieve dedikaryotization (6 to 16 weeks). In large trials with stocks of 24 species of wood pathogenic basidiomycetes (Kerruish and DaCosta 1963), toxic chemicals like sodium arsenate, copper sulphate, sodium dichromate, sodium

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pentachlorophenate, zinc sulphate and boric acid were not able to dedikaryotize any strain. Sodium taurocholate did not prove to be an effective dedikaryotizing agent. It split only one stock of *Coriolus sanguineus* and only one nuclear type was recovered. Similarly, Nishibori and Kinugawa (1978) were able to recover only one nuclear type of various strains of *L. edodes* by using sodium taurocholate. A method for symmetrical dedikaryotization was reported by Leal-Lara and Eger-Hummel (1982) to be effective with *Kuehneromyces mutabilis, Flammulina velutipes* and strains of various *Pleurotus* species (*P. ostreatus, P. cornucopiae* and *P. eryngii*). Dedikaryotization occurred in a medium made of glucose and peptone. Mycelium growth was promoted and dedikaryotization inhibited if the medium was filter sterilized instead of autoclaving and when mineral salts or organic nutrients were added. However, when Arteaga *et al.* (1996) used this technique for dedikaryotization of *Lentinus* spp strains, only one neohaplont type was recovered.

Production of protoplasts have been used recently for dedikaryotization of the edible fungus. Fukumasa *et al.* (1994) used it for dedikaryotization of *L. edodes*. After just seven days incubation, both nuclear types were recovered from 75% of all tested strains. Though neohaplonts showed normal compatibility reactions, considerable variations in other biological characteristics were observed. These variations were attributed to mutation, since similar variations were found among protoclones derived from the parental monokaryotic strains. In later studies, however, the two monokaryotic components were recovered only in certain cases and the proportion of each monokaryotic component among neohaplonts was not always reported. In a study carried out with *L. lepideus*, various factors affecting regeneration and isolation of protoplasts were reported: culture media, lytic enzymes, osmotic regulators for production and regeneration of protoplasts, and optimal pH and incubation time to obtain protoplasts (Kim *et al*. 2000).

Dedikaryotization of commercial strains of *L. edodes* is of great importance and a dedikaryotization method for symmetrical recovery of neohaplonts is urgently required. However, most existing methods for dedikaryotization present serious drawbacks. They are time consuming, produce in most cases an unsymmetrical splitting of the dikaryon and they yield neohaplonts showing considerable deviations from the parental types. In spite of the failure reported for dedikaryotization of *L. edodes* in peptone-glucose solutions, this is a very promising alternative since it avoids the previously-mentioned drawbacks. With *L. edodes* strains of different origins, the effect of modifications in the various stages of the procedure have yet to be tested .

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MATERIALS AND METHODS

Biological material.

Seven strains of *L. edodes* were used: strain L5 from Dr. Ian Reid (National Research Council of Canada), strains L9 and L15 from Dr. Tai-Soo Lee (Forest Research Institute of Korea), strains L18, L19 and L20 from Dr. Rosa L. Andrade (Instituto Tecnológico Querétaro, México) and strain L21 from Amycel (strain 4005). All strains were stored in the culture collection of the Department of Food Science and Biotechnology, Faculty of Chemistry, UNAM.

Culture Media

Malt extract agar (MEA) was prepared dissolving malt extract (15%) and agar (2%) in distilled water. It was sterilized in an autoclave at 121°C for 30 minutes, and 10 ml sterile medium were poured into sterile petri dishes. Plates with solidified medium were wrapped in plastic bags and incubated at 24°C for 2 days to check sterility and then used for storage of strains and propagation of mycelium. Liquid malt extract medium (ME) was prepared by dissolving 1.5% malt extract in distilled water, fifty ml ME was poured in 125 ml Erlenmeyer flasks, which were sterilized in an autoclave at 121°C for 30 minutes.

Dedikaryotization solution

Using the dedikaryotization method recommended for *Pleurotus* spp and *Coprinus* spp, a basic dedikaryotization solution was prepared in distilled water with 2% glucose and 2% peptone P (Oxoid) (Leal-Lara and Eger-Hummel 1982). Fifty ml of this solution were poured into 125 ml Erlenmeyer flasks and sterilized at 121°C for 30 minutes.

Blending of mycelial cultures

Four agar cubes (ca. 0.5 cm) were cut from the edge of a growing colony and distributed on each MEA plate to produce mycelial cultures. After four days incubation at 24°C, colonies reached ca. 4 cm diameter. Fifty ml sterile cold water was poured into a sterile jar of a laboratory blender, and the four colonies were cut and added into the jar. Agar cultures and water were blended at high speed for 5 to 150 seconds. Neohaplonts were recovered from MEA plates and from dedikaryotization solutions inoculated with suspensions of blended agar cultures.

Recovery of neohaplonts from dedikaryotization solution

Flasks with dedikaryotization solution were inoculated with 20 or 50 µl suspension of blended agar cultures and incubated at 24°C. When mycelium growth was visible, 100

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or 200 µl solution were taken to inoculate MEA plates. The solution in the flask was poured into the sterile jar of the blender and then homogenized for five seconds at high speed. MEA plates were inoculated with 20 or 50 µl homogenate and then incubated at 24°C until mycelial growth was noticeable. Mycelium without clamp connections, (monokaryotic type), was identified under the microscope (160x). Each colony was repeatedly cultivated in MEA to verify the absence of clamp connections.

Identification of the two types of neohaplonts

To identify the two component monokaryotic types of a dedikaryotized strain, two neohaplonts were randomly selected and paired in MEA plates with all recovered neohaplonts from this strain. Plates were incubated at 24°C. After 72 hours, plates were regularly inspected under the microscope to identify dikaryotic mycelium. To confirm a correct identification of all neohaplonts, two new neohaplonts were selected and paired again with all available neohaplonts.

RESULTS

In previous work in our laboratory, the dedikaryotization method proposed by Leal-Lara and Eger-Hummel (1982) could not be successfully applied to *L. edodes* strains. Only three of nine strains were dedikaryotized and in all cases only one monokaryotic component was recovered (Arteaga *et al*. 1996). Seven new *L..edodes* strains were processed according to the same method and inoculated in dedikaryotization solutions and MEA plates. After 30 days of incubation, growth was absent in all flasks with dedikaryotization solution and, surprisingly, in all MEA plates as well. This indicated that mycelia of the *L. edodes* strains tested did not survive the first stage of the dedikaryotization method when agar cultures were blended for 150 seconds. Such extreme sensitivity to mechanical damage was confirmed when strain L19 was blended for 120, 90, 60 and 30 seconds, since mycelium growth was again absent in all dedikaryotization solutions. However, in spite of the lack of colonies on MEA plates inoculated with homogenates blended for 120 and 90 seconds, an increased number of colonies were produced on plates from the 60- and 30-second homogenates (Table 1). This sensitivity to blending was confirmed with strains L18 and L19, which showed a considerable increase in mycelial survival upon further reduction of blending time, i.e. 25, 20, 15, 10 and 5 seconds. However, mycelial growth in dedikaryotization solutions was insignificant, even when flasks were inoculated with 5-second homogenates.

Table 1. Effect of blending time of two agar cultures on mycelial development of *Lentinula edodes* strains.

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Strain	Blending of agar cultures (s)	Mycelium development	
		Dedikaryotization solution*	MEA (CFU)
L19	Incubation time = 30 days		
	120	N	1
	90	N	1
	60	N	18
	30	N	36
	Incubation time = 10 days		
	25	N	66
	20	N	55
	15	S	47
	10	S	50
	5	S	43
L18	25	N	59
	20	N	18
	15	S	53
	10	S	34
	5	S	52

* Mycelium development: N = None, S = Scarce

Inoculum volume 20 µl.

The effect of blending time on mycelial survival was evaluated with four additional *L. edodes* strains (Table 2). Strains L5, L9 and L15 proved to be more resistant to blending. However, strains L20 and L21 were able to survive only after short blending times of 5 to 25 seconds For dedikaryotization of very sensitive strains like L21, a larger inoculation volume, e.g. 50 µl should be used to increase the number of viable mycelia. However, the number of colonies obtained with the two inoculation volumes are not proportional. Probably it is not possible to obtain particles of similar size with such blending times, and reproducibility is limited by the rather small inoculation volumes.

Table 2. Effect of blending time of agar cultures on viability of *Lentinula edodes* strains in MEA inoculated with 20 and 50 l.

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Blending of agar cultures (seconds)	CFU in MEA (14 days incubation)									
	Inoculation volume (µl)									
	20					50				
	Strains									
	L5	L9	L15	L20	L21	L5	L9	L15	L20	L21
5	30	10	14	17	1	39	46	62	58	5
10	34	10	31	39	3	20	12	39	67	5
15	8	6	18	3	1	43	8	22	18	7
20	13	0	11	0	0	19	0	26	1	2
25	3	5	8	0	1	18	11	29	2	3
30	14	1	7	0	0	13	3	19	0	2
60	3	1	2	0	0	5	0	7	3	0
90	0	0	2	0	0	1	2	5	2	0
120	0	1	3	0	0	0	0	3	0	1
150	0	0	0	0	0	1	0	1	0	0

Lentinula edodes strains (L5, L9, L15, L20 and L21) were blended 5, 30, 90 and 150 seconds and inoculated either in dedikaryotization solution or in liquid malt extract medium to evaluate whether the composition or type of media, or even mechanical damage affected the ability of *L. edodes* strains to grow. Mycelial death due to mechanical damage by blending was again observed. After 90 seconds blending no growth was found on either liquid media. *L. edodes* mycelium was not able to grow in the rather poor dedikaryotization solution (Table 3). With short blending times, most strains showed a poor mycelial development in dedikaryotization solutions while abundant growth was encountered in ME solutions.

Recovery of neohaplonts had to be attempted by alternative methods, since mycelial growth of *L .edodes* strains in dedikaryotization solutions was hindered by the mortality rate produced when agar cultures were blended. MEA plates were directly inoculated with agar cultures homogenates from various blending times (5 to 150 seconds). Dedikaryotization solutions were also inoculated with five-second homogenates. Once mycelial growth became evident, it was used to inoculate MEA plates. Thereafter, the remaining solution was homogenized for five seconds, and then used to inoculate MEA plates.

From all strains, neohaplonts were recovered after each stage (blending, dedikaryotization solution before and after homogenization). They were classified according to the two monocaryotic components (Type I and II), a third group of a non-compatible type (Table 4). Blending time proved to be crucial for recovery of

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neohaplonts. Recovery was favored when agar cultures were blended for more than twenty seconds, since dikaryotic mycelium predominated with shorter times (data not shown). Neohaplonts were recovered from blended agar cultures in four of the five strains tested. Furthermore, dedikaryotization of strain L20 was achieved only by this method, since it always produced dikaryotic mycelium when inoculated in dedikaryotization solution. For certain strains, recovery of neohaplonts in non-homogenized dedikaryotization solutions was slightly better, and for strain L19, it was the only condition required for recovery of both monokaryotic components. With four strains, a meagre recovery of neohaplonts was attained from homogenized dedikaryotization solutions, and no neohaplonts at all were recovered from the remaining three strains.

Table 3. Effect of blending time of agar cultures on mycelium development of five strains of *Lentinula edodes* in liquid media.

Strains	Blending times-seconds	Mycelium development in liquid media	
		ME	Dedikaryotization solution
L5	5	A	S
	30	-	N
	90	S	N
	150	-	N
L9	5	A	S
	30	-	S
	90	N	N
	150	-	N
L15	5	A	S
	30	-	N
	90	S	N
	150	-	N
L20	5	S	S
	30	-	N
	90	N	N
	150	-	N
L21	5	S	S
	30	-	S
	90	N	N

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	150	-	N

* Mycelium development: N = None, S = Scarce A = Abundant
Media were inoculated with 50 µl agar culture homogenates and incubated 14 days

In order to evaluate if the various strains were symmetrically dedicaryotized, χ^2 values were calculated for the proportion of the two neohaplont types recovered for each strain at the three stages in the dedikaryotization method (Table 5). By comparison to the reference value, symmetrical recovery of both neohaplont types was achieved in all cases with exception of strain L19. Neohaplonts from strain L19 were recovered only from non-homogenized dedikaryotization solution; Type I neohaplonts predominated over those of Type II.

Table 4: Recovery of both neohaplont types at each stage of the dedikaryotization process.

Strains	Stages in the dedikaryotization process						Recovered neohaplonts			
	Blending of agar cultures		Dedikaryotization solution				Total	Non compatible	Types	
			Without homogenization		With homogenization					
	I	II	I	II	I	II			I	II
L5	4	2	7	4	5	4	27	1	16	10
L9	2	6	1	7	0	0	17	1	3	13
L15	0	0	8	8	1	1	21	3	9	9
L18	NT	NT	1	1	0	1	3	0	1	2
L19	NT	NT	15	3	0	0	18	0	15	3
L20	7	15	0	0	0	0	24	2	7	15
L21	4	2	11	6	9	2	34	0	24	10
Total	42		72		23		144	7	75	62

NT: Not tested

Table 5: Evaluation of symmetrical recovery (1:1) of neohaplonts at each stage of the dedikaryotization process.

Strains	χ^2 values for symmetric recovery (1:1)*		
	Stages of the dedikaryotization process		All recovered neohaplonts
	Blending of	Dedikaryotization solution	

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	agar cultures	Without homogenization	Homogenization	
L5	0.66	0.83	0.20	1.38
L9	2.00	4.50	*	6.25
L15	*	0.00	0.00	0.00
L18	NT	0.00	*	0.50
L19	NT	8.00	*	8.00
L20	2.90	*	*	2.90
L21	0.66	1.44	4.16	5.76

χ^2 values less than 6.63 (α= 0.01) indicate a 1:1 ratio for 2 neohaplont types

NT: Not tested

* No recovered neohaplonts

DISCUSSION

This study confirmed the great sensitivity to mechanical damage of *L. edodes* mycelium previously reported by Kawasumi *et al.* (1987). He could not prepare inoculant mycelia of *L. edodes* for protoplast production by fragmentation with a blender, homogenizer or glass beads. It was necessary to cut mycelial cultures into very minute fragments with a thin razor. A combination of fragmentation by razor cutting followed by filtration with a nylon mesh was recommended not only for *L. edodes* but also for other basidiomycetes comparatively difficult to grow in liquid medium. Table 1 shows that homogenization times usually employed for blending of *Pleurotus* spp mycelium (more than 150 seconds) resulted in an extensive death of *L. edodes* mycelium. However, sensitivity to mechanical forces is not similar for all strains (Table 2), Some were extremely sensitive, like strain L21 which barely survived five seconds blending. No strains of *L. edodes* tested in this study, were able to survive blending for more than 30 seconds.

After blending of agar cultures, mycelial damage was so extensive that growth in the peptone-glucose dedikaryotization solution was dramatically diminished (Table 3). The very short times used in this study for blending and homogenization of agar and liquid cultures reduced mycelial damage. Therefore, monokaryotic mycelium of both nuclear types had more chances for survival. This is particularly important when dealing with slow growing or sensitive monocaryons and probably favors recovery of both nuclear types in equal proportions (1:1).

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Any of the factors previously mentioned could explain the failure of previous attempts to dedikaryotize *L. edodes* strains with this method (Arteaga *et al*. 1996). It was also impossible to recover nuclear types by dedikaryotization using toxic chemicals either with *L. edodes* (Takemaru 1964, Nishibori and Kinugawa 1978) or with the other tested species of basidiomycetes. Before this study, successful dedikaryotization of *L. edodes* had been achieved only by Fukumasa *et al*. (1994) with the aid of protoplast production. Both nuclear types were recovered in twelve of the sixteen tested strains; however, they were present in equal proportions in only six of these strains. Such low efficiency (i.e. 37% of all strains) may be the result of the production and regeneration of protoplasts, steps representing stressing situations that affect the component nuclei of the dikaryon to different extents. Both nuclear types were recovered from all tested strains but symmetrical recovery (1:1) was achieved in only 85% of the cases. Nevertheless, it should be noted that considerable variations in different biological characteristics were observed among the recovered neohaplonts. A third group of non-compatible neohaplonts was present in four of the seven *L. edodes* strains dedikaryotized in this study. Nevertheless, such non-compatible monokaryotic strains have also been recovered in our laboratory from meiotic progenies of various species with tetrapolar incompatibility systems (unpublished data). However, non-compatible neohaplonts were present with a low frequency varying according to the strains. Recovery of monokaryotic strains unable to mate probably resulted from unknown factors already present in the parental dikaryons and were not necessarily induced by the dedikaryotization method.

The dedikaryotization procedure utilized in this study is therefore suitable for obtaining the component monokaryons from highly productive *L. edodes* strains. This approach is of great relevance in view of the large variability in the characteristics found among protoplasts populations as well as in basidiospore derived monocaryons (Eichlerová and Homolka 1999, Eichlerová *et al*. 2000).

ACKNOWLEDGEMENTS

This research was supported by grant IN208798 from the Dirección General de Asuntos del Personal Académico (DGAPA) of the National University of Mexico (UNAM)

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