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Optimization of *Pleurotus eryngii* culture parameters and development of improved strains by mating of compatible neohaplonts

Optimización de parámetros de cultivo de *Pleurotus eryngii* y desarrollo de cepas mejoradas por apareamiento de neohaplontes compatibles

C. Alpuche-González[1], B. Ornelas-García[2], H. Leal-Lara[2], R. Villanueva-Arce[1], Y. de las Mercedes Gómez y Gómez[1], M. O. Franco-Hernández[1], M. E. Garín-Aguilar[3], G. Valencia del Toro[1]*

[1]*Laboratorio de Cultivos Celulares de la Sección de Estudios de Posgrado e Investigación, UPIBI, Instituto Politécnico Nacional, Barrio la Laguna s/n Ticomán, CP 07340 México City, México.*

[2]*Departamento de Alimentos y Biotecnología, Facultad de Química, Universidad Nacional Autónoma de México, Cd. Universitaria, 04510 México City, México.*

[3]*Laboratorio de Farmacobiología, FES Iztacala, Universidad Nacional Autónoma de México, Av. de los Barrios No.1. Los Reyes Iztacala, CP 54090 Tlalnepantla, Estado de México, México.*

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Abstract

Improved strains of *P. eryngii* were developed by dedikaryotization of commercial strains and production parameters for its cultivation were optimized for local conditions. A wheat straw (45%) substrate with sawdust (20%), wheat bran (16%) and gluten (5%) packed in 3.5 kg bags produced higher BE (103%) than a cotton waste substrate (BE=74%). Incubation for 21 days was enough and cold treatment or scratching of substrate resulted unnecessary. *P. eryngii* was dedikaryotized by blending mycelium suspensions (300 seconds) and inoculation 50 μL homogenate into solutions with 20 g/L glucose and peptone. In two dedikaryotization experiments, both component monokaryons (neohaplonts) were recovered from strains FQ and MB. Hybrids and reconstructed parental dikaryons were obtained by mating neohaplonts. After the first dedikaryotization experiment, the parental strains produced lower BE (103, 84%) than reconstructed strains (183, 101%) and two hybrids (106, 174%). Following a second dedikaryotization, 17 neohaplonts were mated to produce 3 reconstructed strains and 7 hybrids. Their growth curves were determined on MEA agar and wheat. The Baranyi model showed the best adjustment to growth curves and allowed to separate them into statistically different groups and to calculate the corresponding kinetic parameters (μ_{max}, λ) on MEA agar and wheat.

Keywords: *Pleurotus*, hybrids, dedikaryotization, production.

Resumen

Se desarrollaron cepas mejoradas de *P. eryngii* por dedicariotización de cepas comerciales y se optimizaron los parámetros de producción para condiciones locales. Usando bolsas con 3.5 kg y 21 días de incubación, un sustrato de paja de trigo (45 %) con aserrín (20 %), salvado de trigo (16 %) y gluten (5 %) produjo BE más altas (103 %) que un sustrato con desecho de algodón (BE=74 %). El choque frío o raspado del sustrato resultaron innecesarios. *P. eryngii* fue desdicariotizado al homogenizar micelio (300 segundos) e inocular 50 μL de homogeneizado en soluciones de glucosa y peptona (20 g/L). Se recuperaron ambos componentes monocarióticos (neohaplontes) de las cepas FQ y MB en dos desdicariotizaciones y por apareamientos se obtuvieron híbridos y cepas parentales reconstituidas. En la primera desdicariotización, las cepas parentales produjeron menores BE (103, 84 %) que las cepas reconstituidas (183, 101 %) y los dos híbridos (106, 174 %). En la segunda desdicariotización, de 17 neohaplontes se obtuvieron 3 cepas reconstituidas y 7 híbridos. Sus curvas de crecimiento en agar MEA y trigo mostraron el mejor ajuste con el modelo de Baranyi, siendo separadas en grupos estadísticamente diferentes y calculándose los parámetros cinéticos correspondientes (μ_{max}, λ).

Palabras clave: *Pleurotus*, híbridos, desdicariotización, producción.

* *Corresponding author. E-mail*: gvovaltor@gmail.com

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1 Introduction

The various species of the edible fungus genus *Pleurotus* are cultivated worldwide and with an annual production of 3000 ton / year in 2011 are placed in a second position after Shiitake (*Lentinula* sp.) (Royse, 2014; Martínez-Carrera *et al*., 2016). The species of *Pleurotus* are easy to cultivate and represent an important source of proteins, vitamins and minerals. Among the *Pleurotus* species, however, *Pleurotus eryngii* is preferred by the gastronomic industry since it has a notable taste and texture as well as a long shell life (Manzi *et al*., 2004; Martínez-Carrera *et al*., 2016). Furthermore, it has been used for extraction of polysaccharides (He *et al*., 2016), ligninolytic enzymes (Akpinar and Urek, 2014) and vaccines (Pérez-Martínez *et al*., 2015). Many alternatives for production of *P. eryngii* have been proposed, including different types of substrates, procedures for preparation of substrate, containers for production, incubation time and procedures for induction of fruiting like cold treatment (Xin-Rui *et al*., 2020) or scratching (Alemu, 2014).

A wide variety of agricultural residues have been used for cultivation of *P. eryngii* such as ornamental plants like *Cyperus alternifolius* (Ohga and Royse, 2004) and different types of crop stubble from rice, corn, millet, wheat, beans, soybean, cotton and sugar cane (Okano *et al*., 2007; Kirbag and Akyuz, 2008; Sardar *et al*., 2017). In Asian countries, cultivation of *Pleurotus eryngii* has flourished using cotton waste, a byproduct produced by the cotton textile processing industry. It is a cellulose rich material, locally available in enormous quantities and reportedly to be the best yielding substrate (Babu *et al*., 2007; Adebayo, 2009; Iqbal *et al*., 2018; Khan *et al*., 2019).

Another approach to increase productivity is by developing improved strains. In the conventional procedure, hybrids are produced by pairing monokaryons from spore prints obtained from selected dikaryotic strains. This is a time-consuming technique that does not assure a quick achievement of the desired improved strains. Dedikaryotization of selected dikaryotic strains to obtain their monokaryotic components (neohaplonts) is an effective short cut to produce new dikaryotic combinations with higher probability of being high yielding strains (Eger, 1978; Singh and Kamal, 2017).

Dedikaryotization can be accomplished mechanically or chemically. Leal-Lara and Eger-Hummel (1982) incubated homogenized mycelia in peptone-glycine solutions to induce chemical dedikaryotization of various *Pleurotus* species. Both types of the component monokaryons were recovered from all dikaryotic strains, even in the case of a sporeless dikaryon. In recent studies, dedikaryotization of native *Pleurotus* strains from Oaxaca was reported by Guadarrama-Mendoza *et al*. (2014), Sánchez-Hernández *et al*. (2019) produced hybrids after dedikaryotization of *P. djamor* and Valenzuela-Cobos *et al*. (2020b) obtained inter genera hybrids after dedikaryotization of various *Pleurotus* and *Lentinula* strains. Dedikaryotization of *P. eryngii* was firstly reported by Ramírez-Carrillo *et al*. (2011), the recovered *P. eryngii* neohaplonts were paired with neohaplonts obtained by dedikaryotization of various strains of *L. edodes* to produce inter-genera hybrids, which showed increased productivity, i.e., biological efficiency (BE) and a wide variety of morphological characteristics.

This study aims to establish the conditions for a successful commercial production of *P. eryngii* in México. Two approaches are proposed: a) parameters for commercial production must be optimized according to local conditions (types of substrates and materials available for cultivation) b) dedikaryotization of two commercial *P. eryngii* strains to produce hybrids and to reconstruct the parental strains and their production on commercial substrate. Determination of the kinetic parameters (μ_{max} and λ) on solid growing media of hybrids, reconstructed and parental strains is an additional activity to be done. This could be a valuable information to establish a strategy for identification of high producing strains (BE).

2 Materials and methods

2.1 Strains

The two commercial *P. eryngii* strains, FQ and MB, and the reconstructed and hybrid strains used in this study are stored in the culture collection of Laboratorio de Cultivos Celulares, Unidad Profesional Interdisciplinaria de Biotecnología (UPIBI-IPN).

2.2 Culture media and fructification

All *P. eryngii* strains used in this study were grown in Petri dishes with 1.5 % malt extract agar (MEA).

Grain spawn was prepared by soaking wheat grain overnight in excess water. After draining excess water, 150 g of soaked wheat grain were filled in polypropylene autoclavable bags and sterilized at 121 °C, 15 lb/in^2 for 1 hour. After cooling, the sterilized grain was inoculated with full grown agar plates and incubated for 15-20 days at 25 °C.

Preparation of substrates. Substrate ingredients (Table 1) were mixed, and water was added to achieve 67 % water content. Wet substrates were filled into polypropylene autoclavable bags with 2 or 3.5 kg substrate and autoclaved at 121 °C, 15 lb/in^2 for 3 hours. The following day, bags with sterilized substrate were inoculated with 5 % (w/w) of the previously prepared grain spawn and incubated for 35, 28 or 21 days at 25 °C.

Fruiting of substrates. The full-grown substrates were liberated from the upper part of the bag and placed on racks at a spacing of 15-20 cm in the fruiting room at 18-19 ºC, 75 - 85% air humidity and continuous ventilation so that CO_2 concentration were less than 700 ppm.

Table 1. Substrates for cultivation of *P. eryngii* (composition in dry weight).

Ingredients	F	M	N	A	B	C
Cotton Waste	72	-	-	-	-	-
Sawdust	-	44	20	30	25	34
Wheat straw	10	30	45	40	50	34
Wheat bran	5	16	22	15	15	20
Gluten	5	5	7	10	5	7
$CaCO_3$	6	5	6	5	5	5
$CaSO_3$	2	-	-	-	-	-
Total	100	100	100	100	100	100

In certain cases, before the bag was opened, full grown substrates were subjected to cold treatment (2 ºC for 24 h) or to scratching (i.e., the surface of the substrate was rubbed with the inner part of the closed bag and incubated for another 24 hours). The fruiting substrates were watered, when necessary, to maintain them moist. Fruit bodies were cropped from substrates in an early stage of maturation, i.e., when the pileus cap was beginning to open. The weight of fresh mushrooms and number of mushrooms produced from each replica was registered to calculate production (P) (g of fresh mushrooms /bag) and biological efficiency (BE) (g of fresh mushrooms per 100 g of dry substrate) of each strain.

2.3 Dedikaryotization

Dedikaryotization solution was prepared in 50 mL flasks with 20 g/L anhydrous glucose (G) Merck and 20 g/L peptone P (P) (Oxoid). The mycelium of a full-grown MEA plate was cut in pieces of approx. 1 cm^2 and then poured in a waring blender with 50 mL of sterile distilled water and then blended at maximum speed for 300 s. The flasks with 50 mL dedikaryotization solution were inoculated with 50 μL of the resulting homogenate and incubated at 24 °C. When mycelium growth became visible, the whole content of the flask was then homogenized for 30 s and MEA plates were inoculated with 25 or 50 μL of this homogenate and incubated at 28 °C. Developing colonies were microscopically (10x and 40x) examined to identify monokaryotic colonies, i.e., without clamp connections (Valencia del Toro and Leal-Lara, 1999; 2002; Sánchez-Hernández *et al*., 2019). Such colonies were isolated by transferring to new MEA plates and further evaluated for absence of clamp connections, and then considered as neohaplonts.

2.4 Identification of compatibility types

To evaluate compatibility, a piece of agar 0.5 cm^2 was cut from a full-grown culture and placed together on a MEA plate with another piece of agar with mycelium from another culture and then incubated at 28 °C. Clamp connections, indicating different compatibility types, were detected by daily inspection under the microscope (10x y 40x) (Valencia del Toro and Leal-Lara, 1999; 2002; Sánchez-Hernández *et al*., 2019). By this way, the two neohaplont types were identified for each dedikaryotized *P. eryngii* strain, FQ and MB. Each positive pairing, i.e., clamp formation, indicated that the parental strain has been reconstructed.

2.5 Production of hybrid strains

Following the procedure previously described, neohaplonts from strain FQ were paired with neohaplonts from strain MB. The compatible pairings, those resulting in clamp formation, corresponded to the production of a dikaryotic hybrid strains capable of fruiting (Guadarrama-Mendoza *et al*., 2014; Sánchez-Hernández *et al*., 2019; Valenzuela-Cobos *et al*., 2017).

2.6 Mycelial growth kinetics

Growth curves of parental, reconstructed and hybrid strains on MEA and wheat solid media were determined with ten replicates. Colony diameter of agar plates was measured daily until full colonization of the petri dish (Regina, 2001). Glass tubes with sterilized wheat grains (2 h at 121 °C and 15 lb/in^2) were inoculated at the upper part and the advance of the mycelium front towards the bottom was daily measured. The volume of wheat in each tube was determined using following equation:

$$V = h\pi r^2 \tag{1}$$

Where V = mycelium growth volume (cm^3), h = distance advanced by mycelium front (cm), π = 3.1416, r = tube radius (cm).

To calculate kinetic parameters like maximum growth rate (μ_{max}) and lag phase (λ) on wheat and MEA agar, data of growth curves were adjusted to various models (lineal, Baranyi, exponential and logistic) to identify the model with the lowest mean absolute error (MAE) (Valenzuela-Cobos *et al*., 2017).

Baranyi model:

$$y(t_{max}) = y_{max} + \ln\left(\frac{-1 + e^{\mu_{max}*\lambda} + e^{\mu_{max}*T}}{-1 + e^{\mu_{max}*T} + e^{\mu_{max}*\lambda + y_{max} - y_0}}\right) \tag{2}$$

λ = lag phase, μ_{max} = maximum growth rate, y_{max} = growth rate at the last day, y_0 = growth rate at the first day, T = days, e = 2.718281, $y(t_{max})$ = growth rate.

Exponential model:

$$y(t_{max}) = y_{max} + \mu_{max}(T - \lambda) - \ln(e^{y_{max} - y_0} - 1 + e^{\mu_{max}*T(T-\lambda)}) \tag{3}$$

λ = lag phase, μ_{max} = maximum growth rate, y_{max} = growth rate at the last day, y_0 = growth rate at the first day, T = number of days, e = 2.718281, $y(t_{max})$ = growth rate.

Logistic model:

$$y(t_{max}) = \frac{A}{1 + \exp(4\mu_{\max}/A * (\lambda - T) + 2)} \quad (4)$$

A = model parameter, λ = lag phase, $\mu_{\max}$ = maximum growth rate, T = number of days, $y(t_{max})$ = growth rate.

Differences in the growth curves of parental, reconstructed and hybrid strains were evaluated by ANOVA analysis of all data, from day 1 to day 10 or day 15. When differences were found, Post Hoc Duncan test (α = 0.05) was used to separate statistically different groups.

3 Results and discussion

3.1 *Optimization of culture parameters of Pleurotus eryngii*

The lack of consensus about optimal parameters for a successful production of *P. eryngii*, acquires relevance in Mexico since this fungus has not been produced commercially. Local conditions and availability of raw materials for preparation of substrates differ greatly from other areas where *P. eryngii* has been extensively cultivated (Chang and Miles, 1989). Thus, optimization of cultivation parameters for local conditions is of utmost importance.

3.1.1 *Incubation time, cold treatment and cotton waste in substrate*

For good yields, long incubation times of substrates have been recommended by different authors (Stamets, 2005; Shen, 2017). Likewise, cold treatment of full-grown substrates for induction of fruiting (Stamets, 2005) and the use of cotton waste in substrate for good yields by *P. eryngii* has also been widely recommended (Khan *et al*., 2019; Xie *et al*. 2016). To evaluate these parameters, two commercial strains (FQ, MB) were grown on two substrates, cotton waste and a mixture of sawdust with wheat straw. Substrates were incubated for 28 or 35 days and subjected to cold treatment (Table 2).

Table 2. Effect of incubation time, *cold treatment (2 ºC for 24 h) and cotton waste in substrate (2 kg bags) on biological efficiency and average weight of *P. eryngii* (strain FQ).

Substrate	Incubation time (days)	Production	
		Biological efficiency (%)	Average weight (g)
F	28	74.2 ± 9.7	43.9 ± 10.7
F	35	62.9 ± 7.2	42.0 ± 9.0
F*	35	62.5 ± 9.9	38.0 ± 7.0
M	35	58.6 ± 8.6	37.7 ± 6.3

Substrate F (72 % cotton waste), Substrate M (44 % sawdust; 30 % wheat straw)

Biological efficiency was 74.2 % with 28 days incubation, i.e., 11 % higher than with 35 days (62.9 %) but no significant difference was established. Therefore, yields are not decreased when incubation time is reduced from 35 to 28 days. Similarly, no significant differences were found when substrate was subjected to cold treatment (62.5 %) or when cotton waste is substituted by sawdust in substrate (58.6 %). Regarding average weight, it was in the range of 37.7 up to 43.9 g but no significant difference was also observed due to incubation time, cold treatment, or substrate composition.

Although Stamets (2005) recommends cold treatment for promoting fruiting with various fungi like *Flammulina velutipes* and various *Pleurotus* species thriving in cold climates, our results did not show any benefits. Similar results were observed by Wang *et al*. (2018), which used an 8-day cold treatment at 4 °C with 2 parental strains and 14 hybrids of *Pleurotus tuoliensis*, they reported biological efficiencies of 40.1 and 46.3 % for parental strains and 37.4 to 49.4 % for hybrid strains. Xin-Rui *et al*. (2020) reduced to 6 days the cold treatment at 4 °C reporting BE within 41.8 - 49.3 % for *P. tuoliensis*. So, cold treatment did not affect BE with *P. tuoliensis*, however, in our study with *P. eryngii* cold treatment produced a decrease in BE values. Similarly, Ramírez-Carrillo and Leal-Lara (2002) obtained very high BE, 143 to 261 %, with *L. edodes* without using cold treatment. Therefore, cold treatment is not necessarily recommended to increase yields.

P. eryngii has been grown on cotton waste with good results. Iqbal *et al*. (2018) and Sardar *et al*. (2017) reported 72 % BE with a 100 % cotton waste substrate while Khan *et al*. (2019) obtained 93 % BE when glycine betaine was added to cotton waste. According to Iqbal *et al*. (2018) the high yields from cotton waste was associated to its high cellulose. However, the mixture of sawdust (44 %) with wheat straw (30 %) in substrate M is a good substitute for cotton waste since yields were similar (Table 2).

3.1.2 *Production of P. eryngii on wheat straw-based substrates*

Since a substrate of sawdust with wheat straw produced similar yields as cotton waste, the effect of different proportions of sawdust and wheat straw were studied in the next experiment (Table 3). Substrates were incubated for 28 days, and cold treatment was not used.

Substrate N produced the highest BE (103 %), almost double than substrates B, A, C (63, 55, 53 %). Substrate N is rich in nutrients since it contains high amounts of wheat bran and straw while the lowest proportion of sawdust and it compares favorably to other attempts to substitute cotton waste. Xin-Rui *et al*. (2020) reported 44.65 to 53.2 % BE in a substrate of cotton seed hulls (60 %), sawdust (23 %), wheat bran (10 %) and corn starch (5 %) and Moonmoon *et al*. (2010) reported 73.5 % BE in a substrate of sawdust (64 %), wheat bran (32 %) and cotton seed hulls 4 %.

Table 3. Accumulated production and average weight of *P. eryngii* (strain FQ) on wheat straw-based substrates.

Substrate	Accumulated production				Average weight (g)		
	Biological Efficiency (%)		g fresh mushrooms / bag				
	Week 1	Week 2	Week 1	Week 2	Week 1	Week 2	Global
N	81 ± 1	103 ± 4	536 ± 6	683 ± 25	25 ± 1	31 ± 1	26 ± 1
A	54 ± 8	55 ± 7	356 ± 54	365 ± 49	25 ± 1	21 ± 2	25 ± 2
B	63 ± 14	63 ± 14	417 ± 93	417 ± 93	34 ± 8	-	34 ± 8
C	53 ± 24	53 ± 24	348 ± 156	348 ± 156	29 ± 8	-	29 ± 8

Incubation: 28 days, Bags: 2 kg. Mushrooms cropped from day 1 to day 7 are quoted under week 1 and those cropped from day 8 to day 14 are quoted under week 2.

Table 4. Effect of bag size (kg) and incubation time on accumulated production and average weight of *P. eryngii*.

Strain	Incubation (days)	Bag (kg)	Accumulated production				Average weight (g)		
			Biological Efficiency (%)		g fresh mushrooms / bag				
			Week 1	Week 2	Week 1	Week 2	Week 1	Week 2	Global
FQ	21	2	101 ± 7	107 ± 9	663 ± 49	709 ± 58	30 ± 2	30 ± 3	29 ± 2
FQ	21	3.5	115 ± 2	126 ± 3	1325 ± 21	1455 ± 32	36 ± 3	31 ± 1	34 ± 1
FQ	28	2	81 ± 1	103 ± 4	536 ± 6	683 ± 25	25 ± 1	31 ± 1	26 ± 1
FQ	28	3.5	77 ± 5	123 ± 4	890 ± 53	1421 ± 42	32 ± 2	33 ± 1	32 ± 1
MB	28	2	84 ± 12	84 ± 12	553 ± 81	553 ± 81	34 ± 9	-	34 ± 9
MB	28	3.5	16 ± 2	89 ± 4	185 ± 18	1026 ± 44	32 ± 5	29 ± 2	30 ± 1

Substrate: N. Mushrooms cropped from day 1 to day 7 are quoted under week 1 and those cropped from day 8 to day 14 are quoted under week 2.

Rodriguez Estrada *et al*. (2009) obtained higher yields (114.8 - 132.8 %) but using a commercial supplement (Remo's, corn and soybean based) in a substrate of cotton seed hulls (56 %), *Quercus rubra* sawdust (27 %), ground soybean (12 %) and corn distiller's waste (4 %).

3.1.3 Effect of bag size and incubation time on production by P. eryngii

In previous studies for production of *P. eryngii*, substrate has been packed in small units (bags) equal or less than 1 kg of substrate like Moonmoon *et al*. (2010) and Xin-Rui *et al*. (2020). Only Rodriguez Estrada *et al*. (2009) used containers with 2.5 kg of substrate. Maintaining BE in bags with increasing amount of substrate would favorably impact productivity. Therefore, bags with 3.5 kg and 2 kg of substrate were compared (Table 4) with two different commercial strains of *P. eryngii* (FQ, MB). The effect of reducing incubation time from 28 to 21 days was also tested.

Bags with 3.5 kg substrate showed higher yields than 2 kg bags with strain FQ (126 *vs* 107 % BE for 21 days incubation and 123 *vs* 103 % BE for 28 days incubation). Such effect was not observed with strain MB for 28 days incubation (Table 4). According to Hernández *et al*. (2003) and Mandeel *et al*. (2005) desiccation of substrate is normally reduced in bags with larger quantity of substrate, thus bags with 3.5 kg allowed higher BE. For commercial production of *Pleurotus* spp, bags with more substrate are generally recommended, i.e., 4 - 5 kg by Gaitán-Hernández *et al*. (2006) and Castañeda and Leal (2007), 6 - 7 kg by Soto-Velazco (2007) and even 10-12 kg by Muez-Orobia and Pardo-Nuñez (2001). Further experimentation has to be carried out to elucidate if productivity with *P. eryngii* is maintained using containers/bags with even larger amounts of substrate.

Decreasing incubation time from 28 to 21 days did not affect yield of strain FQ, i.e., 103 *vs* 107 % BE (2 kg bags) and 123 *vs* 126 % BE (3.5 kg bags). Such high BE with 21 days incubation compares favorably with previous studies. Ohga and Royse (2004) reported 100 - 125 % BE in substrates incubated for 30 days, Rodriguez Estrada *et al*. (2009) obtained 114.8 - 132.8 % BE after incubating supplemented substrates for 25 days and Xin-Riu *et al*. (2020) obtained 44.65 - 53.2 % BE and incubated for 5 additional days after complete mycelium colonization.

3.1.4 Effect of scratching on production by P. eryngii

Scratching of surface mycelia in full grown substrates has been recommended for induction of fruiting and to obtain good yields with different species of edible fungi (Stamets, 2005; Shen, 2017; Liu *et al*., 2018; Ha *et al*., 2020). Accordingly, various authors used this procedure for production of *P. eryngii* (Kim *et al*., 1997; Rodríguez Estrada *et al*., 2007; Kim *et al*., 2008; Andrino *et al*., 2011), and BE in the range of 41 - 87 % have been reported. However, production of *P. eryngii* in substrates subjected to scratching has not been compared with non-scratched substrates. Therefore, the effect of scratching of substrate on BE was evaluated in bags with 3.5 kg substate and 28 days incubation (Table 5).

Table 5. Effect of scratching of substrate on production and average weight of *P. eryngii* (strain MB).

Scratching	Production		Average weight (g)
	Biological Efficiency (%)	g fresh mushrooms / bag	
Yes	16.4 ± 7.8	189.8 ± 90.3	38.3 ± 8.3
No	47.1 ± 5.9	544.1 ± 68.0	30.2 ± 2.7

Substrate: M, Incubation: 28 days, Bags: 3.5 kg.

Table 6. Recovered neohaplonts from strains FQ and MB of *P. eryngii* (1st Experiment).

	Strain FQ		Strain MB	
	Type I	Type II	Type I	Type II
	1	17	2	19
Total	18		21	

Table 7. Mating of neohaplonts of strains FQ and MB of *P. eryngii* (1st Experiment). Hybrids (H) and reconstituted (R) strains are showed.

		Strain FQ		Strain MB	
		A_3B_3	A_4B_4	A_3B_2/A_2B_3	A_1B_1
		bFQ1	bFQ5	bMB10	bMB17
A_3B_3	bFQ1	-	+(RBFQ)	-	+(HB2)
A_4B_4	bFQ5		-	+(HB3)	-
A_3B_2/A_2B_3	bMB10			-	+(RBMB)
A_1B_1	bMB17				-

Table 8. Accumulated production and average weight of reconstituted, hybrid and parental strains of *P. eryngii*.

Strain	Accumulated production				Average weight (g)		
	Biological Efficiency (%)		g fresh mushrooms / bag				
	Week 1	Week 2	Week 1	Week 2	Week 1	Week 2	Global
RBFQ	24 ± 2	183 ± 4	156 ± 13	1206 ± 27	33 ± 1	37 ± 1	37 ± 1
RBMB	9 ± 2	101 ± 2	58 ± 12	667 ± 16	52 ± 15	32 ± 1	33 ± 2
HB2	18 ± 4	106 ± 6	121 ± 29	700 ± 40	43 ± 5	31 ± 2	33 ± 2
HB3	15 ± 1	174 ± 10	97 ± 5	1148 ± 64	26 ± 3	35 ± 1	34 ± 1
FQ	81 ± 1	103 ± 4	536 ± 6	683 ± 25	25 ± 1	31 ± 1	26 ± 1
MB	84 ± 12	84 ± 12	553 ± 81	553 ± 81	34 ± 9	-	34 ± 9

Substrate: N, Incubation: 28 days, Bags: 2 kg. Mushrooms cropped from day 1 to day 7 are quoted under week 1 and those cropped from day 8 to day 14 are quoted under week 2.

Scratching of substrate resulted in a lower BE (16 %) than control, non-scratched substrates (47.1 %). Although Stamets (2005) recommended scratching of substrates for promoting porosity of substrates and to induce fruiting, with *P. eryngii* such effect was not observed.

3.2 Production of hybrid strains by dedikaryotization

3.2.1 Production of hybrid strains and determination of productivity on straw substrates

Dedikaryotization of selected dikaryotic strains for recovery of the component monokaryons to generate new hybrid strains is an approach that allows to by-pass meiosis (Sánchez-Hernández *et al*., 2019; Singh and Kamal, 2017). So, two commercial *P. eryngii* strains (FQ, MB) were subjected to dedikaryotization (Table 6).

The two monokaryotic components were recovered for each strain, however, one monokaryotic component (neohaplont) surpassed the other component in both cases, 19 *vs* 2 for strain MB and 17 *vs* 1 for strain FQ. Arteaga-Santillan *et al*. (1996) reported asymmetrical recovery of neohaplonts for various strains of *Lentinula* spp., suggesting the component monokaryons presented different susceptibility to experimental conditions of the dedikaryotization procedure. Accordingly, Valencia del Toro and Leal-Lara (2002) reported the monokaryotic

components of various *Pleurotus* spp. strains were affected differently by peptone-glucose concentrations and mechanical treatment (Valencia del Toro and Leal-Lara, 2002).

To identify mating types, one neohaplont from each monokaryotic type was selected for strain MB and for strain FQ. They were mated crosswise as shown in Table 7, confirming that a common mating type was present in both strains. Simultaneously, strains FQ and MB were reconstructed (R), i.e., RBFQ (BFQ1×BFQ5) and RBMB (BMB10×BMB17), and two hybrids (H) were obtained, i.e., HB2 (BFQ1×BMB17) and HB3 (BFQ5×BMB10).

Reconstructed strains and the two hybrids were fruited in 2 kg bags of substrate N with 28 days incubation. Table 8 shows BE and average weight for 2 weeks production. Surprisingly, reconstructed strains showed higher BE than parental strains, i.e., 183 *vs* 103 % for strain FQ and 102 *vs* 84 % for strain MB. Notably, hybrid HB3 produced a higher BE (174 %) than parental strains. These 3 strains represent a strain improvement for commercial production of *P. eryngii*.

Production of hybrids and reconstruction of parental strains by chemical dedikaryotization of different *Pleurotus* species has already been reported. Valenzuela-Cobos *et al*. (2019) found no difference in BE between a parental *P. ostreatus* strain and its reconstructed strain (104.2 *vs* 98.2 %) though with a *P. djamour* strain, a lower BE was produced by the reconstructed strain (141.8 *vs* 106.7 %). In a later study, Valenzuela-Cobos *et al*. (2020a) did not observe differences between parental and reconstructed strains of *P. ostreatus* when grown in 2 different substrates (95.6 *vs* 92.2 % in substrate WS and 72.2 *vs* 78.9 % in substrate AP) while reconstructed strains of *P. djamour* showed higher yields (110.3 *vs* 125.8 % in substrate WS and 86.34 *vs* 98.4 % in substrate AP). A conventional approach to obtain improved strains was used by Salmones *et al*. (2020); out of spore prints from 3 different *P. pulmonarius* parental strains (with BE 91.9, 102.9 and 114.8 %) 43 hybrids were obtained and the BE reported for 13 hybrids were in the range of 81.4 to 147.5 %. However, the high BE produced in our experiment by the reconstructed strain RB-FQ (183 %) and hybrid HB3 (174 %) are superior to those referred by previous reports.

A different approach to produce hybrids and to reconstruct parental strains was reported by Chakraborty & Sikdar (2007), in this case by producing protoplasts to obtain neohaplonts. No differences in BE were registered between the reconstructed and parental strains of *P. florida* and *V. volvacea* and just slight differences in morphological characteristics were reported, i.e., stipe and pileus size.

3.2.2 *Production of hybrid strains and determination of mycelial kinetics on solid media*

Dedikaryotization of strains FQ and MB was again tried to evaluate if dedikaryotization of these strains is always asymmetrical. It would be feasible to obtain a larger number of neohaplonts allowing a clear confirmation of mating types and again, to reconstruct parental strains and to generate a larger number of hybrids. In this second dedikaryotization experiment, 30 g/L peptone and glucose concentration was used and as Table 9 shows, neohaplonts were again recovered asymmetrically from strain FQ (4 *vs* 18) and from strain MB (1 *vs* 3).

Table 9. Recovered neohaplonts from strains MB and FQ of *P. eryngii* (2nd Experiment).

	Strain FQ		Strain MB	
	Type I	Type II	Type I	Type II
	4	18	1	3
Total	22		4	

This second experiment confirmed that one mating type of each strain, i.e., FQ and MB, is extremely sensitive to the dedikaryotization process, so that it is recovered in very low numbers. Among others, blending of mycelial suspensions (time, speed), glucose and peptone concentrations and inoculation volume may be important as has been reported for other basidiomycetes (Guadarrama-Mendoza *et al*., 2014; Valenzuela-Cobos *et al*., 2020a). Compatibility patterns of the neohaplonts from strain FQ and MB confirmed the presence of a common compatibility factor in both strains, which limited an extensive hybridization of all neohaplonts from these strains. As shown in Table 10, a total of 7 hybrids and 3 reconstructed strains were recovered to study their growth kinetics.

Mycelial growth on solid media has been proposed as an important factor for selection of strains for mushroom production. Clark and Anderson (2004) proposed that those strains colonizing substrate in a short time will fruit quickly and with higher yields. So, the selected strains were grown on Petri dishes with malt extract agar (MEA) and in flasks with wheat grains, for 10 and 15 days, respectively.

ANOVA analysis of all data of the growth curves (from day 1 to day 10 or day 15) shown on Figure 1, indicated significant differences among parental reconstructed and hybrid strains. According to Duncan test, 5 groups were stablished for growth curves on MAE (Figure 1A), hybrid HC7 (group E) was the fastest growing strain, followed by hybrids HC1, HC3, HC4, HC5 and HC8 (group D). Noteworthily, parental strains were classified in slower growing groups, i.e., MB in group C and FQ in group B, and its 3 reconstructed strains were scattered in different groups: RCFQ2 (group C), RCFQ3 (group B) and RCFQ1 (group A).

The corresponding classification of the growth curves on wheat media (Figure 1B) shows a somewhat different picture. Even though the fastest growing strain was a hybrid, HC1 (group D), the parental strains FQ, MB were placed together with hybrids HC2, HC4, showing better growth than 4 of the other hybrids (HC3, HC5, HC7 and HC8). Remarkably, in this case, the 3 reconstructed strains: RCFQ1, RCFQ2, RCFQ3, were the slower growing strains (group A).

Table 10. Mating of neohaplonts of strains FQ and MB of *P. eryngii* (2nd Experiment). Hybrids (H) and reconstituted (R) strains are shown.

		Neohaplonts 2nd Experiment																
		Strain FQ													**Strain MB**			
		A_3B_3									**A_4B_4**				**A_3B_1/A_1B_3**	**A_2B_4/A_4B_2**		
		cFQ1	**cFQ2**	**cFQ4**	**cFQ5**	**cFQ7**	**cFQ10**	**cFQ15**	**cFQ17**	**cFQ18**	**cFQ12**	**cFQ35**	**cFQ37**	**cFQ40**	**cMB6**	**cMB1**	**cMB8**	**cMB10**
	cFQ1		-	-	-	-	-	-	-	-	+	+	**+RCFQ3**	**+RCFQ2**	-	+	+	+
	cFQ2			-	-	-	-	-	-	-	+	+	**+RCFQ1**	+	-	+	+	+
	cFQ4				-	-	-	-	-	-	+	+	+	+	-	**+HC1**	**+HC4**	+
	cFQ5					-	-	-	-	-	+	+	+	+	-	**+HC2**	**+HC5**	+
A_3B_3	**cFQ7**						-	-	-	-	+	+	+	+	-	+	+	+
	cFQ10							-	-	-	+	+	+	+	-	**+HC3**	+	+
	cFQ15								-	-	+	+	+	+	-	+	+	+
	cFQ17									-	+	+	+	+	-	+	+	**+HC7**
	cFQ18										+	+	+	+	-	+	+	**+HC8**
	cFQ12											+	+	+	+	-	-	-
A_4B_4	**cFQ35**												+	+	+	-	-	-
	cFQ37													+	+	-	-	-
	cFQ40														+	-	-	-
A_3B_1/A_1B_3	**cMB6**															+	+	+
	cMB1																-	-
A_2B_4/A_4B_2	**cMB8**																	-
	cMB10																	

These results highlight that the different strains did not show a consistent pattern of growth on distinct solid media. Therefore, it is of utmost importance to characterize the growth of strains on the different solid media according to suitable indicators of its performance. Different kinetic growing models have to be used for determination of kinetic parameters like maximum growth specific speed (μ_{max}) and lag phase (λ), to select the model which fits best to the experimental data. With such information, a correlation between kinetic parameters and productivity (BE) could be eventually drawn. Although fruiting is a complex process dependent upon different factors, of environmental and genetic nature, this information could deliver an empirical approach for identification of strains with improved productivity.

Various models (lineal, Baranyi, exponential and logistic) were evaluated to identify the model with the lowest mean absolute error (MAE). The lowest MAE was obtained with the Baranyi model for both solid media, 7.59 % for wheat and 0.60 % for agar (MEA) (Table 11). Valenzuela-Cobos *et al*. (2020a y 2020b) reported quite different results. For wheat the lowest MAE was shown by the exponential (1.03 %) and the logistic (4.14 %) models while for agar media (MEA) the logistic and linear model showed the lowest MAE (2.52 and 1.67 %, respectively), and noteworthy, the Baranyi model showed the highest MAE in opposition to our results, probably as a result that different species were used by Valenzuela-Cobos *et al*. (2020b), i.e., *P. ostreatus* and *P. djamor*.

The kinetic parameters, μ_{max} and λ, calculated according to the Baranyi model are shown on Table 11 for wheat and agar media (MEA). The classification previously established (groups) for the corresponding growth curves (Figure 1) are also indicated. On agar media. hybrid strain HC7 (cMB10xcFQ17) was the fastest growing (μ_{max} = 8.6 mm/day) and it was followed by a group (D) of 5 hybrids, i.e., HC8, HC1, HC5, HC3 and HC4, while the parental strains showed the lowest μ_{max} (6.9 and 4.5 mm/day). Strikingly, the 3 reconstructed strains of parental strain FQ, showed differences in the growth curves (Figure1), their μ_{max} varied widely, from 3.5 up to 6.9 mm/day. Thus, the characteristics from the parental strains are extensively modified in the reconstructed strains. Some type of epigenetic phenomena could be playing a role in this situation. The marked differences in yields produced by the reconstructed strains observed in Table 8 could be explained by this way. Although no differences between parental and reconstructed strains of *P. ostreatus* were found in previous

studies by Valenzuela-Cobos *et al*. (2020a) (μ_{max} = 8.7 *vs* 9.15 mm/day) and by Valenzuela-Cobos *et al*. (2020b) (μ_{max} = 9.6 *vs* 10.2 mm/day), our results indicate that a large number of reconstructed strains have to be tested to evaluate possible changes in reconstructed strains. This situation is confirmed by the results of Valenzuela-Cobos *et al*. (2020a) that reported significant differences in μ_{max} (9.6 *vs* 10.2 mm/day) between parental and reconstructed *P. djamor* strains.

The results obtained on wheat differ somehow to those obtained on agar media. In this case, hybrid HC1 (cMB1xcFQ4) was the fastest growing strain (35.3 cm^3/day) and the parental strains were placed in the second group (C) with μ_{max} = 33.0 and 30.8 cm^3/day, respectively while hybrid HC7 (cMB10×cFQ17), the fastest growing strain on agar media, was placed in this case, in the third group (B) with μ_{max} = 29.5 cm^3/day. Noteworthy, in this case the 3 reconstructed strains of parental strain FQ showed the lowest μ_{max} (22.4 to 26.4 cm^3/day) of all strains (group A) validating that the characteristics of the parental strains are extensively modified in the reconstructed strains. These results confirm that it is possible to obtain improved hybrids strains by pairing neohaplonts recovered by dedikaryotization of parental strains.

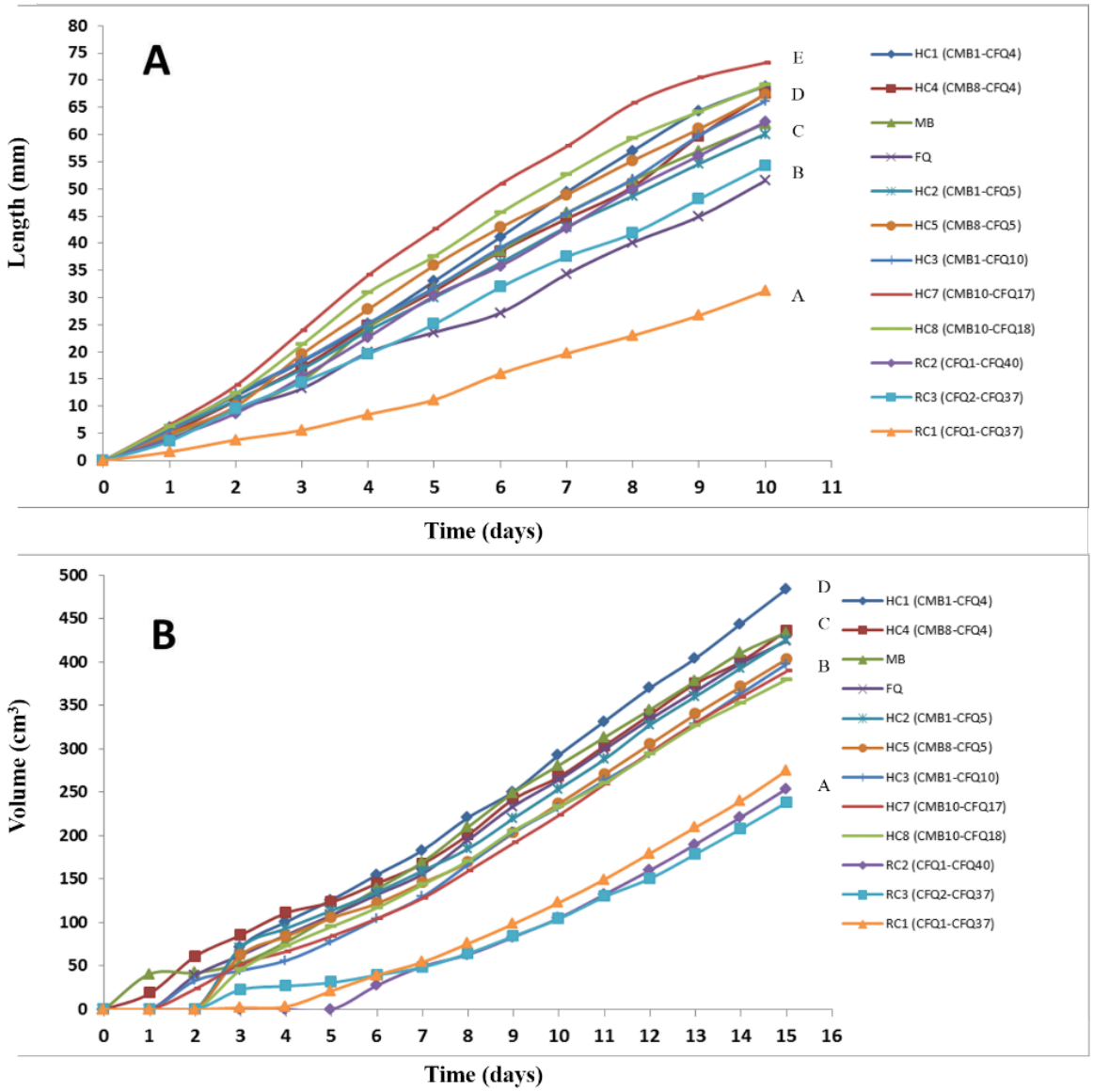

Fig. 1. Mycelium growth curves for parental, reconstituted and hybrid strains in Petri dishes with MEA (A) and in flasks with wheat grains (B).

Table 11. Growth parameters (lag phase = λ and growth rate = μ_{max}, according to the Baranyi model) of parental, reconstituted and hybrid strains on MEA agar plates and flasks with wheat grain. Strains are ordered according to the classification (Duncan groups) of the growing curves on wheat and agar.

Strain	MEA		Duncan Group	Wheat		Duncan Group
	λ (days)	μ_{max} (1/days)		λ (days)	μ_{max} (1/days)	
MB	1.5	6.9	C	2.9	33.0	C
FQ	2.5	4.5	B	1.3	30.8	C
RC1 (cFQ1xcFQ37)	1.7	3.5	A	5.2	26.4	A
RC2 (cFQ1xcFQ40)	1.3	6.9	C	5.4	25.0	A
RC3 (cFQ2xcFQ37)	1.0	5.8	B	5.7	22.4	A
HC1 (cMB1xcFQ4)	0.4	7.5	D	1.6	35.3	D
HC2 (cMB1xcFQ5)	0.2	6.3	C	1.5	30.9	C
HC4 (cMB8xcFQ4)	0.3	6.7	D	0.5	29.2	C
HC3 (cMB1xcFQ10)	0.2	6.7	D	2.8	30.6	B
HC7 (cMB10xcFQ17)	0.2	8.6	E	2.5	29.5	B
HC5 (cMB8xcFQ5)	0.4	7.4	D	1.7	29.4	B
HC8 (cMB10xcFQ18)	0.2	7.8	D	1.8	28.6	B

The time required by a strain to adapt to the growing media is described by the other kinetic parameter λ (Chatterjee *et al*., 2015). Noteworthy, on MEA agar media all hybrids showed lower λ values (0.2 to 0.4 days) than parental (1.5 and 2.5 days) and reconstructed (1 to 1.7 days) strains. Again, the picture on wheat media is somehow different and did not correspond to the pattern observed on MEA agar, i.e., the reconstructed strains presented notably higher λ values (5.2 to 5.7 days) than the parental strains (1.3 and 2.9 days) while λ values for hybrids were scattered from 0.5 to 2.8 days.

Although growing curves could be adjusted to the Baranyi model and classified in statistically different groups, the marked differences in the kinetic parameters (μ_{max} and λ) on MEA agar and wheat do not allow to draw conclusions about the behavior of the different strains on a lignocellulosic substrate as far as their capacity to colonize it and to produce mushrooms. Therefore, it is of highest importance to evaluate the productivity (BE) of the strains obtained after this second dedikaryotization experiment (Table 11) to evaluate a possible correlation with the kinetic parameters, μ_{max} and λ. If such correlation could be stablished, an important advance could be achieved to obtain strains with new characteristics.

Conclusions

Various parameters for production of *Pleurotus eryngii* were optimized. High BE were obtained in bags with 3.5 kg bags of substrates where cotton waste has been substituted by a mixture of wheat straw, sawdust and wheat bran. Incubation times could be reduced to 21 days, and it was stablished that cold treatment or scratching of substrate was not necessary.

Successful dedikaryotization of *P. eryngii* (strains FQ and MB) was achieved in this study by blending mycelium suspensions for 300 seconds and 50 μL of this homogenate were inoculated into solutions with 20 g/L glucose and peptone. Upon appearance of mycelium, the mycelial cultures were again blended for 60 seconds and 25 μL of this homogenate inoculated in MEA plates. In two different experiments, although dedikaryotization was not symmetrical, both neohaplonts types were recovered and mating types clearly identified. By pairing compatible neohaplonts, the parental dikaryons were reconstructed and hybrid strains were recovered.

In a first experiment, the reconstructed parental strains and two hybrids produced higher yields than the corresponding parental strains. In a second dedikaryotization experiment with a larger number of neohaplonts, the parental strains were reconstructed, and a larger number of hybrids produced. Growth curves of the various types of strains were adjusted to various models (lineal, Baranyi, exponential and logistic). The Baranyi model showed the lowest mean absolute error (MAE) and allowed to separate growth curves in statistically different groups and to calculate kinetic parameters, μ_{max} and λ. Although direct selection of productive strains on lignocellulosic substrates is not possible with this information, an important advance has been achieved with this study.

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