Induction of Chlamydospores in *Pleurotus pulmonarius* (Grey Oyster Mushroom) as Inoculum and Development of Lyophilized Spawn

by

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LIST OF ABBREVIATIONS

μCT	x-ray microtomographic
A.	*Agaricus*
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
BMP	Bitmap image file
C.	*Candida*
$CaCl_2.2H_2O$	Calcium chloride dihydrate.
CCD	Charge-coupled device
CT	Computed tomographic
D-	Dextrose
DICOM	Digital Imaging and Communications in Medicine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNS	Dinitrosalicylic acid
FITS	Flexible Image Transport System
GIF	Graphics Interchange Format
IFA	Immunofluorescence assay
IGS	Intergenic Spacer region
ITS	Internal transcribed spacer
JPEG	Joint Photographic Experts Group
MEA	Malt Extract Agar
Na_2SO_4	Sodium sulfate
NaCl	Sodium chloride
NIH	National Institutes of Health

P'-*p*-DDT	Dichlorodiphenyltrichloroethane
P.	*Pleurotus*
PDA	Potato Dextrose Agar
PEG-6000	Polyethylene glycol 6000
PNG	Portable Network Graphics
PSD	Photoshop design file
R.	*Rhizobium*
R^2	R-squared
rRNA	Ribonucleic acid
S	Substrate
SEM	Scanning electron microscope
Sp.	Species
SSU	Small subunit ribosomal
TIFF	Tagged Image File Format
UNIX	Operating system
UV-Vis	Ultraviolet–visible spectroscopy
V.	*Volvariella*

LIST OF SYMBOLS

%	Percent
±	Plus-minus
≤	Smaller than
≥	Larger than
°C	Degree Celcius
µg/ml	Microgram per mililitre
µmax	Maximum growth rate
C	y intercept
Cm	Centimetre
d^{-1}	Per day
dx/dt	Velocity
g	Gram
$g.L^{-1}$	Gram per litre
h^{-1}	Per hour
KPA	Kilopascal
K_s	Contois constant
L	Litre
L^{-1}	Per litre
M	Slope of the graph
mg/gm	Miligram per gram
ml	Microlitre
Mm	Micrometre
mM	Milimolar

N	Normality
Nm	Nanomolar
Rpm	Rotation per minutes
U	Unit
w/v	Weight over volume
y	Y coordinate

Induksi Klamidospora dalam *Pleurotus Pulmonarius* (Cendawan Tiram Kelabu) sebagai Inokulum dan Perkembangan Benih Beku Kering

ABSTRAK

P. pulmonarius ditanam dengan menggunakan benih yang disuntik dengan kultur miselium yang diperoleh dari spora atau kultur tisu. Walau bagaimanapun, penggunaan spora berisiko untuk terdedah kepada pencemaran manakala penggunaan kultur tisu boleh menyumbang kepada perubahan somaklonal. Benih cendawan boleh hanya dalam masa 30 hari. Oleh itu, kajian ini dijalankan untuk meningkatkan pengeluaran dan teknik pemeliharaan untuk benih *P. pulmonarius* dengan menggunakan kombinasi teknik pendorongan sporulasi dan pembeku keringan kaedah pendorongan sporulasi. Klamidospora telah didorong dengan menggunakan pelbagai media tekanan iaitu D-Glukosa Kanji Larut, D-Glukosa $CaCl.2H_2O$, D- Glukosa NaCl, D- Glukosa PEG6000, D- Glukosa Gliserol, and D- Glukosa Na_2SO_4. Tiga parameter iaitu; pH, suhu, dan tempoh inkubasi telah dipilih untuk pengoptimuman penghasilan klamidospora. Kajian telah mendapati bahawa, penggunaan medium tekanan D-Glukosa Kanji Larut menunjukkan pengeluaran klamidospora tertinggi sebanyak 4.5×10^7 dicapai selepas 78 jam, pada pH 6 dan 26˚C suhu dengan menggunakan 1.5×10^7 saiz inokulum. Seterusnya, benih tersebut dirawat dengan 20% campuran trehalosa dan susu skim sebagai pelindung pembeku keringan dan dibeku keringkan dengan menggunakan pembeku keringan (Labconco) pada -40ºC untuk 2 hingga 3 hari. Benih tersebut ditutup dan disimpan pada suhu bilik untuk 1 bulan. Selepas itu, benih tersebut dirawat dengan 2 jam rawatan rehidrasi. Benih klamidospora-dibeku keringkan kemudiannya dibandingkan dalam aspek kadar pertumbuhan dan morfologi dengan benih klamidospora yang diawet dengan menggunakan proses seperti krioawetan, sub-kultur, dan teknik penyejukan 4˚C. Analisis pertumbuhan kinetik mendapati bahawa benih klamidospora mempamerkan kadar pertumbuhan yang lebih tinggi iaitu $0.24d^{-1}$ berbanding benih dari kultur tisu. Benih klamidospora yang dibeku keringkan juga menunjukkan kadar pertumbuhan yang tinggi berbanding benih yang dihasilkan menggunakan teknik pemeliharaan lain dengan $0.154d^{-1}$. Pemerhatian morfologi pada jana buah dari benih klamidospora menunjukkan kuantiti (14.3±1 jana buah), saiz (10.1±1 cm), berat basah (122±6 g), dan peratusan kawasan zarah kelabu (51.34±2%) yang lebih tinggi berbanding jana buah dari kaedah kultur tisu dengan pengeluaran dan kualiti jana buah dapat dikekalkan bagi 3 bulan jangka hayat. Saiz dan berat basah jana buah dari benih klamidospora yang dibeku keringkan juga tidak menunjukkan perbezaan yang signifikan dengan rawatan lain yang membuktikan bahawa benih *P. pulmonarius* dapat dibeku keringkan. Penemuan ini membuktikan penggunaan klamidospora sebagai inokulum boleh dijadikan sebagai pengganti yang lebih baik untuk penggunaan spora dan kultur tisu, manakala teknik pembeku keringan berpotensi untuk pemeliharaan benih *P. pulmonarius*. Ini merupakan hasil kajian pertama ke atas pendorongan sporulasi terhadap spesis *P. pulmonarius*.

Induction of Chlamydospores in *Pleurotus Pulmonarius* (Grey Oyster Mushroom) as Inoculum and Development of Lyophilized Spawn

ABSTRACT

P. pulmonarius is cultivated by using spawn inoculated with mycelial cultures obtained from spore or tissue culture. However, the application of spore is prone to contamination while tissue culture application may contribute to somaclonal variation. The mushroom grain spawn can be only stored for 30 days. Hence, this study was conducted to improve production and preservation technique for *P. pulmonarius* spawns by using the combination of induce asexual sporulation and lyophilization techniques respectively. The chlamydospore was induced using various stress media namely D-Glucose Soluble Starch, D-Glucose $CaCl.2H_2O$, D-Glucose NaCl, D-Glucose PEG6000, D-Glucose Glycerol, and D-Glucose Na_2SO_4. Three parameters namely; pH, temperature, and incubation period were selected for optimization of chlamydospore formation. It was found that, D-glucose soluble starch stress medium showed the highest 4.5 $x10^7$ chlamydospore production with highest chlamydospore production achieved after 78 hours, at pH 6 and 26˚C of temperature by using 1.5 x 10^7 inoculum size. Next, the spawn was pre-treated with 20% mixture of trehalose and skimmed milk lyoprotectant and lyophilized by using freeze dryer (Labconco) at -40ºC for 2 to 3 days. The spawn was sealed and stored at room temperature for 1 month. After that, the spawn was treated with 2 hours of rehydration treatment prior to inoculation on PDA medium. The chlamydospore-lyophilized spawn was then compared with the chlamydospore spawn which preserved using cryopreservation, sub-culturing, and 4˚C refrigeration techniques in aspect of growth rate and yield. Growth kinetic analysis found that chlamydospore spawn exhibited higher growth rate; $0.24d^{-1}$ compared to tissue culture spawn. Lyophilized chlamydospore spawn also shows high growth rate compared the spawn produced using other preservation techniques with 0.154 d^{-1}. Morphological observation on fruiting body of chlamydospore spawn shows higher quantity (14.3±1 sporophore), size (10.1±1 cm), wet weight (122±6 g), and grey particle area percentage (51.34±2%) compared to tissue culture fruiting body with maintained mushroom quality and production for 3 month shelf life compared to tissue culture spawn. Fruiting body size and wet weight of lyophilized chlamydospore spawn shows no significant different with other treatments that indicates that *P. pulmonarius* spawn are lyophilizable. These findings prove that the chlamydospore used as inoculum can serve as better substitute to spore and tissue culture application, whereas lyophilisation technique as potential preservation method for *P. pulmonarius* spawn. This is also the first research ever conducted on induce asexual sporulation of *P. pulmonarius*.

CHAPTER 1

INTRODUCTION

1.1 Background

In the old centuries, mushrooms are one of the earliest food source for human being (Daba *et al.*, 2011). However, they are only consumed by the nobles and rich peoples (Kumari and Achal, 2008). But now, mushrooms are consumed by people from all over the world (Daba *et al.*, 2011). They are preferred due to their delicacy and also its high medicinal value (Asghar *et al.*, 2007; Barros *et al.*, 2007; Rathee *et al.*, 2011). Their function is not only limited as a food source, but also widely applied in pharmaceutical production and in the agriculture field (Khader, 2005; Daba *et al.*, 2011; Nidadavolu *et al.*, 2012). In pharmaceutical industries, bioactive compound from mushrooms are extracted out, processed, and produced as medicines and also additional supplements (Kumari *et al.*, 2012; Nidadavolu *et al.*, 2012).

Nowadays, there are numerous varieties of edible mushrooms which are cultivated world wide to satisfy people's need especially in food industries (Sánchez, 2010; Mukhopadhyay and Guha, 2015). One of the most preferred and top ranked edible mushrooms is *P. pulmonarius* (Chiu *et al*., 1998) (Figure 1.1). It is preferred due to its excellent flavor and taste, high nutritional and high medicinal value (Lavi *et al*., 2012).

Figure 1.1: *P. pulmonarius*

Currently, the rate of mushrooms demand has exceeded the rate of local mushroom supply or production (Department of Agriculture,Malaysia, 2010; Mohd Zaffrie and Azahar Harun, 2015; Rosmiza *et al*., 2016). In order to fulfill the demands of mushrooms, our country have imported high amount of mushrooms from abroad especially from Thailand, China, and also Hong Kong. Based on statistics derived from Department of Agriculture Malaysia (Table 1.1), the amount of imported mushrooms is higher than the number of exported mushroom from our country (Department of Agriculture,Malaysia, 2010; Mohd Anim, 2014; Rosmiza *et al*., 2016). This indicates high demands of mushroom in Malaysia and low local production which is unable to fullfill the demand of mushroom in our local market.

The rate of imported mushroom increased from year to year indicates that the demand of the mushroom increased tremendously (Mohd Zaffrie and Azahar Harun, 2015). The high demand of mushroom in our local market is also supported by the data

showed in Figure 1.2 and Figure 1.3 by which mushroom is enlisted as one of the agriculture product that requires 10.1% increment of mushroom production in year 2020. The increased in estimated mushroom production in year 2010-2020 indicates the importance of mushroom cultivation in Malaysia (Department of Agriculture, Malaysia, 2010). High production of mushroom helps to fullfill the high demand of mushroom and reduce the importation of mushroom from foreign countries (Mohd Zaffrie and Azahar Harun, 2015; Rosmiza *et al*., 2016). Moreover, Malaysia Agricultural Policy 2014 also enlisted mushroom as industrial type of plantation or cultivation and also include mushroom in agrotourism plan (Department of Agriculture, Malaysia, 2010; Mohd Zaffrie and Azahar Harun, 2015; Rosmiza *et al*., 2016). This indicates the importance of mushroom cultivation in our country and the production of mushroom especially *P. pulmonarius* needed to be increased.

Table 1.1: Statistics data on mushroom export and import in Malaysia. (Mohd Anim, 2014)

Year	Import	Export
2001	1,810 million tan (RM 16.99 Million)	168 million tan (RM 3.95 Million)
2005	3,034 million tan (RM 32.07 Million)	134 million tan (RM 1.55 Million)
2007	21, 077 million tan (RM 86.44 Million)	4,805 million tan (RM 33.45 Million)
2009	17, 036 million tan (RM 91.04 Million)	4,949 million tan (RM 43.20 Million)
2010	17,277 million tan (RM 83.43 Million)	3,6288 million tan (RM 30.13 Million)
2011	20,513 million tan (RM 116.1 Million)	3,255 million tan (RM 33.1 Million)