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Teck Nam Ang, Dr.
Gek Cheng Ngoh, Dr, University of Malaya
Adeline Seak May Chua, Dr, University of Malaya

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Development of a novel inoculum preparation method for solid-state fermentation – Cellophane Film Culture (CFC) technique

Teck Nam Ang  Gek Cheng Ngoh *  Adeline Seak May Chua

Department of Chemical Engineering, Faculty of Engineering, University of Malaya, 50603
Kuala Lumpur, Malaysia.

* Corresponding author.

Postal address: Department of Chemical Engineering, Faculty of Engineering, University of Malaya, 50603 Kuala Lumpur, Malaysia. Telephone: +6 03-7967 5286; fax: +6 03-7967 5371; e-mail address: ngoh@um.edu.my

Abstract

This study reports a user-friendly technique in the preparation of fungal inoculum intended for solid-state fermentation (SSF) – Cellophane Film Culture (CFC) technique. This technique uses cellophane film-overlaid agar plates to facilitate the separation of fungal biomass. The findings showed that inoculum of P. sajor-caju produced is viable, and it was confirmed by the presence of laccase enzyme activity in SSF of rice husk. The correlation between fungal dry and wet weights ($r^2 = 0.9329$) provides an accurate estimation of fungal
dry weight from its wet weight during inoculum preparation. Besides, this technique does not require a strict sterile handling condition and possesses lower risk of contamination compared with liquid culture and agar plugs approaches. In consideration of large scale inoculum preparation, this newly developed technique is comparatively more cost-effective, which further suggests its potential in inoculum preparation from mycelial fungi for SSF.

**Keywords**

*Inoculum; solid-state fermentation; cellophane; fungus; rice husk.*

**1. Introduction**

Solid-state fermentation (SSF) has received enormous attention for its biological and processing advantages compared to liquid and submerged fermentations (Holker et al., 2004). To date, SSF studies were conducted at laboratory scale with only few at pilot scale due to unresolved technological and operational constraints particularly when mycelial fungus is employed in the process. One of the constraints is the preparation of inoculum.

In current practice, inoculum for SSF is prepared by growing mycelial fungus in liquid culture or by taking plugs from actively expanding end of an established culture (Gupte et al., 2007; Matsubara et al., 2006). Unlike unicellular bacteria and yeast, inoculum preparation by growing fungus in liquid culture is troublesome due to the changes of its physiological state in liquid. The fungus tends to clump and distribute unevenly in liquid culture; thus, this complicates the quantification of fungal biomass. The fungal plug method too has limitation in quantifying fungal biomass as inoculum can only be quantified discretely by the number of plugs introduced in SSF. Furthermore, nutrient agar attaching to fungal biomass in the plugs could be an inherent source of contaminations.
In view of the abovementioned issues, an alternative inoculum preparation method that can overcome the limitations is desirable. This has led to the development of a simple and effective method to separate fungal biomass from the cellophane film-overlaid agar plate and to alleviate the related contamination problems in this work. The thin transparent cellophane film is made of plant-based cellulose, and it is semi-permeable to moisture. The film can be autoclaved and withstand heat up to 190°C making it suitable for sterile microbiological works. Most importantly, it is biodegradable and does not have negative impact on the environment.

The purpose of the present study is to examine the feasibility of the newly developed cellophane film culture technique (hereafter named CFC technique) in inoculum preparation, and subsequently to validate the viability of the inoculum prepared in SSF. In addition, the performance of CFC technique in inoculum preparation was also evaluated.

2. Materials and methods

2.1. Inoculum preparation and regression analysis

_Pleurotus sajor-caju_ (DSM8265), which was screened for ligninolytic enzyme activities (Ang et al., 2011), was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The inoculum of _P. sajor-caju_ was prepared by transferring 6-mm-diameter agar plugs from an established culture to malt extract peptone agar (MEPA) plates overlaid with autoclaved cellophane films (refer Fig. A1 in Supplementary Data). The inoculated plates were incubated at 25°C in the dark. The fungal biomass was separated from the cellophane film for the determination of fungal wet weight and the respective dry weight was determined after drying at 80°C overnight as outlined by Reeslev and Kjoller (1995).
total of 57 replicates were performed, and sampling was carried out from the third day of incubation onwards until complete colonization of the plates. For regression analysis, the fungal wet and dry weights were fitted to a linear model with linear regression by using SigmaPlot for Windows Version 11.0 (Systat Software, Inc., Germany).

2.2 Technique verification

Verification of the technique was carried out via SSF of rice husk pretreated according to Ang et al. (2012), and the viability of the inoculum was measured in terms of laccase activity. The conditions of SSF were: initial moisture level of substrate, 55% (w/w); inoculum loading, 0.5% (w/w); fermentation duration, 10 days; incubation temperature, 27°C; and pH of the moistening agent, pH 7. At the end of SSF, laccase enzyme was harvested with approximately 13 ml 100mM citrate buffer (pH 4.8), and the enzyme filtrate was stored at -20°C prior to analysis.

The laccase activity was assayed by monitoring the increment in absorbance from the oxidation of guaiacol at 470 nm with Secomam Prim Advances UV-VIS spectrophotometer at 25°C using a molar extinction coefficient ($\varepsilon_{470}$) of 6,740 M$^{-1}$cm$^{-1}$ (Jhadav et al., 2009). The absorbance ($A$) is directly proportional to the concentration of guaiacol oxidation product formed (Beer’s Law) and the thickness of the sample (Lambert’s Law) and their relationship is represented by the Beer-Lambert equation,

$$A = \varepsilon \cdot c \cdot l$$

(1)

, where $\varepsilon$ = molar extinction coefficient, $c$ = concentration of guaiacol oxidation product in mol/L, and $l$ = thickness of the sample in cm. The enzyme activity is expressed in U/ml,
which is defined as the amount of enzyme catalyzing the production of one µmol coloured
product per minute per millilitre.

For comparison, *P. sajor-caju* was grown on MEPA plates overlaid with nylon
membrane, where it has been used to grow mycelial fungus for microbiological studies
(Funder & Johannessen, 1957). A total of 20 replicates were carried out in the study.

2.3. Technique validation

The validity of the inoculum preparation technique was conducted by using *Pycnoporus*
sanguineus (DSM3023), which has also been reported for its laccase production via SSF
(Abdul Karim & Mohamad Annuar, 2009). A total of 20 replicates were carried out in the
validation study.

3. Results and discussion

3.1. Inoculum preparation and regression analysis

Estimation of fungal dry weight is essential during inoculum preparation as it is frequently
used to report inoculum loading in SSF studies. The technique developed in this study offers
a quick estimation of dry weight of fungal biomass while maintaining the viability of the
inoculum. The application of cellophane film prevents penetration of hyphae into agar
medium, and makes the separation of fungus for biomass determination possible. Figure 1a
illustrates the correlation plot of dry to wet weight of *P. sajor-caju* cultivated on agar plates
overlaid with cellophane film. The linear regression has a coefficient of determination ($r^2$) of
0.9329, which explains ~93% variability of the relationship between the fungal dry and wet
weights. The regression equation exhibits a higher accuracy for fungal wet weight below 0.8
g (equivalent to incubation period of 6 to 8 days). As the weight of fungal biomass increases,
the estimation of fungal dry weight becomes less accurate due to the absorption of water on the biomass. However, this does not reduce the reliability of the technique as inoculum is usually harvested at day 7 when fungus is actively growing.

Figure 1: Regression plots of fungal dry and wet weights.
3.2. Technique verification and validation

Inoculum prepared via this technique gave a desired laccase activity (5.03 U/L) in SSF of rice husk. The findings indicate that separation of fungus from the cellophane film did not damage the fungus as the fungus was striving on the rice husk.

For comparison purpose, *P. sajor-caju* was also grown on MEPA plates overlaid with nylon membrane. Figure 1b depicts the regression plot of dry to wet weights of *P. sajor-caju* cultivated on agar plates overlaid with nylon membrane. The regression plot possessed a high $r^2$ (0.9634), which was slightly higher compared to the $r^2$ exhibited by the fungus grown on cellophane film. In spite of this, the use of nylon membrane in inoculum preparation has some disadvantages. It incurs higher cost than cellophane film, especially when production of a large volume of inoculum is involved.Besides, separation of fungal biomass from nylon membrane is difficult due to its relatively porous surface structure compared to cellophane film. Furthermore, the biomass of *P. sajor-caju* grown on nylon membrane was half of the fungal biomass grown on cellophane film for a same incubation period (refer Fig. 1a and b). The difference in fungal biomass density observed between the two overlaying materials might be attributed to the fungal physiology on different growing surfaces.

Further validation of the technique was conducted by using another strain of white-rot fungus (*P. sanguineus*). The findings showed that *P. sanguineus* can also grow on cellophane film and its regression plot shown in Figure 1c exhibits a good correlation of fungal dry to wet weights.
3.3. Advantages of CFC technique

Added advantages to the verified and validated CFC technique are its ability to produce actively-growing inoculum and its association with lower risk of contamination compared to liquid culture approach. The actively-growing fungus from this proposed technique permits rapid colonization of the solid substrate following inoculation as it has been adapted to grow on solid surface during the preparation stage. This could be demonstrated by the fungus exhibiting homologous morphological appearance when it was grown on the rice husk and the agar plate that overlaid with cellophane film as shown in Figure 2a and 2b, respectively. In contrast, the fungus has a distinctive morphology when it was grown in liquid state, where the mycelial fungus assumes a globular shape in the absence of solid support (Fig. 2c and 2d). Fungal inoculum grown in liquid state might result in a longer colonization time (48 – 72 hours) after inoculation into SSF substrate due to changes in the fungal physiology associated to the difference in morphology when it is grown in different states. Preparation of fungal inoculum in liquid culture requires strict sterility and possesses higher risk of contamination (refer Fig. A2 and A3 in Supplementary Data for fungal growth in liquid culture and illustration of liquid culture contamination). Furthermore, nutrient medium absorbed in the interstitial space of the fungal globular structure is a potential source of contamination, where it may attract contaminating microbes especially when SSF is conducted under non-aseptic condition. Another method involves direct inoculation of fungal plugs with nutrient agar, wherein the latter also shows higher risk of contamination like the liquid culture approach.
Figure 2: Morphology of *P. sajor-caju* grown on (a) rice husk, (b) agar plate, and in (c, aerial view; d, bottom view) liquid culture.

From the perspective of scaling-up, preparation of inoculum using liquid culture approach becomes intricate when involving large quantity of inoculum, which requires costly and sophisticated bioreactor. However, the CFC technique developed allows preparation of large quantity of inoculum in a simple manner by sub-culturing the fungus on more agar plates overlaid with cellophane films. As long as the agar medium and cellophane films are sterilized and handled aseptically during the process, this technique can produce good quality inoculum. With respect to the sterility of the autoclaved cellophane films on agar plates, it can be maintained up to a period of 12 months or longer at room temperature. In addition to that, the transfer of inoculum from agar plates to solid substrate can be performed without the need of a laminar flow cabinet.
4. Conclusions

A user-friendly and cost effective technique for the preparation of fungal inoculum was developed in this study. The cellophane film culture (CFC) technique has added advantages over liquid culture and agar plugs approaches as it requires less stringent aseptic operating condition, while presents a lower risk of contaminations. Moreover, this technique has the potential to produce viable inoculum in large quantity, which enhances the feasibility of this technique for inoculum preparation in SSF.

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References


Supplementary Data

Inoculum Preparation

Cellophane film culture (CFC) technique uses malt extract peptone agar (MEPA) plates overlaid with autoclaved cellophane films for the preparation of fungal inoculum. Figure A1 illustrates a cellophane film is being overlaid on an agar plate.

![Fig. A1. Overlaying agar plate with cellophane film in cellophane film culture (CFC) technique.](image)

Inoculum Preparation via Liquid Culture Approach

The liquid culture of *Pleurotus sajor-caju* was carried out in malt extract broth inoculated with three agar plugs of established culture, and it was incubated in an orbital shaker (110 rpm) at 25°C. The time profile of fungal weight was conducted by taking 20 ml of ‘evenly’ mixed sample from the flask at 24-hour interval for 7 days. The fungal biomass was filtered with a pre-weighed 0.45 µm membrane filter, and dried at 80°C until constant weight was observed. Figure A2 shows the time profile of *P. sajor-caju* in the liquid culture, which requires ~42 hours of lag period before it reaches maximal growth after ~108 hours of incubation. The fungus clumped into globular structure when it was growing in liquid state.

![Fig. A2. Time profile of *P. sajor-caju* growth in malt extract broth.](image)
In the preparation of fungal inoculum using liquid culture approach, contamination was observed in some of the flasks regardless of aseptic condition was maintained during preparation and incubation stages. Figure A3 shows an example of liquid culture contamination.

**Fig. A3.** Observed contamination of liquid media during inoculum preparation. The liquid cultures containing 3% malt extract broth were inoculated with 3 fungal agar plugs, and incubated on an orbital shaker (110 rpm) at 25°C.