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# Acceleration of fungal spore production by embedding a hydrophobic polymer net in a nutrient agar plate

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ABSTRACT

A simple and novel procedure for the acceleration of fungal spore production was developed. A net of hydrophobic polymer such as polypropylene (PP) and polytetrafluoroethylene (PTFE) was embedded in a nutrient agar plate, and effect of the polymer net on spore production by 6 fungal strains, such as *Aspergillus terreus*, *Penicillium multicolor*, and *Trichoderma virens* were estimated. The effect of hydrophobic polymer net was insufficient in a liquid-surface immobilization (LSI) system with fungal cells immobilized on a ballooned microsphere layer formed on a liquid medium surface. On the other hand, the embedding of a PTFE net in an agar plate remarkably enhanced the spore production in all 6 strains tested to produce 2.0–8.5 × $10^7$ spores/$cm^2$-agar plate surface. Especially, the spore production by *A. terreus* ATCC 20542 in the presence of a PTFE net was 7.7 times as much than that in no net. Positive correlations between the hydrophobicity of net and the spore production were observed in all 6 strains ($R^2$, 0.653–0.999).



## 1. Introduction

Fungal spores are useful for the biocontrol agent exterminating some plant pathogens and harmful insects in addition to used as seed inoculum for fermentation industries (Brimner and Boland, 2003; Khan et al., 2012). For example, *Trichoderma atroviride* spores have effective biocontrol activity against many plant pathogens such as *Rhizoctonia solani* (Daryaei et al., 2016), *Phytophthora* spp (Bae et al., 2016), and *Guignardia citricarpa* (De Lima et al., 2016). On the other hand, entomopathogenic *Beauveria bassiana* spores exhibit strong insecticidal activity toward many harmful insects, such as *Rhynchophorous ferrugineus* (Dembilio et al., 2010), *Corcyra cephalonica* (Kaur et al., 2014), and *Dermanyssus gallinae* (Immediato et al., 2015).

On the other hand, it is known that many fungal spores have many active enzyme systems and metabolic pathways (Murata, 1993; Wolken et al., 2003). For example, while the spores of *Aspergillus niger* (Kulkarni et al., 1998), *Aspergillus ochraceus* (Dutta and Samanta, 1999), and *Cunninghamella elegans* (Jaworski et al., 2007) were used as biocatalysts for biotransformation of steroids in an aqueous phase, other *A. niger* and *Penicillium digitatum* were used for terpenoid conversion in an aqueous phase (Demyttenaere et al., 2000; Wolken and van der Werf, 2001). Thus, spores are anything but inactive dormant cells like a lot of people are thinking, but they have fully many enzyme systems and can catalyze various microbial transformations.

Recently, the authors have developed two novel non-aqueous bioconversion systems with fungal spores (Oda et al., 2018). It is well known that fungal spores exhibit the excellent tolerance against may kinds of stresses, such as dryness and high temperature states, UV-irradiation, and many antifungal chemicals (Guarro et al., 1997; Wolken et al., 2002; Hedgecock et al., 2010). The authors have also confirmed the superior tolerance of fungal spores against various organic solvents (Oda et al., 2014). Therefore, it is strongly expected that the fungal spores can be applied as tough biocatalysts to non-aqueous or organic–aqueous two-liquid-phase systems (Oda et al., 2018).

Concerning the production of fungal spores, both submerged (SmC) (Issaly et al., 2005; Jackson et al., 1997) and solid-state cultivation (SSC) (Krasniewski et al., 2006; Viccini et al., 2007) have been widely used. In the SSC system, it is well known that initial water content substantially affects the production of fungal spores (Aregger, 1992; Parra et al., 2004) together with other conditions, such as some supplementary nutrients (Devi, 1994;

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Krasniewski et al., 2006) and kinds of solid substrates (Prakash et al., 2008; Larena et al., 2004). Thus, it is suggested that various factors significantly affect the production of fungal spores in the SSC system.

Recently, the authors have found that charged state of a fungal cell–ballooned microsphere layer in two interface bioprocesses significantly affects hydroxylation activity and secondary metabolite production. The two processes are extractive liquid-surface immobilization (Ext-LSI; fermentation system) system (Oda, 2017; Oda and Isshiki, 2007) and liquid–liquid interface bioreactor (L–L IBR; bioconversion system) (Oda, 2017; Oda et al., 2009). The processes comprise a hydrophobic organic solvent (upper phase), a fungal mat with ballooned microspheres (middle phase), and a liquid medium (lower phase).

In the two systems, the addition of anion-exchange resin micro-pieces into the middle phase of the L-L IBR and the Ext-LSI systems improved alkane-hydroxylation activity by *Monilliera* sp. NAP 00702 (Oda et al., 2012a) and production of secondary metabolite by *T. atroviride* AG 2755-5NM398 (Oda et al., 2012b), respectively. Interestingly in the later case, spore production was remarkably repressed by adding anion-exchange resins (Oda et al., 2012b) in the Ext-LSI system.

On the other hand, addition of hydrophobic resin micro-pieces into the middle phase of the L–L IBR enhanced the subterminal hydroxylation activity of *n*-decane by *Monilliera* sp. NAP00702 without significant morphology change containing spore formation (Oda et al., 2013). However, as for other fungal strains, the authors expected that physicochemical properties, especially the hydrophobicity, affected on the spore formation by fungi growing on the surfaces of a ballooned microsphere layer and an agar plate.

In this study, effect of the hydrophobic environment in the LSI and the agar plate cultivation systems on the spore production was estimated with 8 fungal strains. Through the above trials, a novel, economical, and efficient procedure for the acceleration of fungal spore production was developed.

## 2. Materials and methods

### 2.1. Microorganisms and their preservation, restoration

Eight available fungi, *Aspergillus sojae* NBRC 32074, *Aspergillus oryzae* RIB40, *Aspergillus brasiliensis* NBRC 6341, *Aspergillus terreus* ATCC 20542, *Penicillium griseofulvum* NBRC 7641, *P. chrysogenum* NBRC 4626, *P. multicolor*, IAM 7153, and *Trichoderma virens* NBRC 6355 were used. Concerning the preservation of each strain, a fungal mat was formed on the surface of a potato-dextrose agar (PDA; pH 6.0) plate prepared in a PP tube (8 mm i.d., 2 mL), and highly branched hydrocarbon, 2,6,10,15,19,23-hexamethyltetracosane (squalane; $C_{30}H_{62}$; Mw, 422.83), was added onto the fungal mat. The fungal strains were reserved at 4 °C. Squalane may be used as an alternative organic solvent to liquid paraffin because the highly branched solvent may be resistant toward the attack by fungal enzymes such as cytochrome P450 monooxygenase, excellent biocompatibility, high oxygen solubility, drying preservation ability, and very low volatility.

All strains stocked were restored on a PDA at 25 °C. For the preparation of seed broth, all strains were inoculated in F-1 medium consisted of 20.0 g of potato starch, 10.0 g of glucose, 20.0 g of soy protein (Soypro™; Inui Co. Ltd., Osaka), 1.0 g of $KH_2PO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 1.0 L of reverse osmosis water, pH 6.0. Cultivation was done at 25 °C with shaking (200 rpm) for 3 d.

### 2.2. Construction of LSI system set a hydrophobic polymer net

Twenty mL of potato dextrose broth diluted by 2 times, 1.3 g of porous microsphere (Advancel HB-2051, Sekisui Chemical Co. Ltd., Tokyo), and 400 μL of a spore suspension were vigorously mixed. The microsphere is prepared from polymethylmethacrylate (PMMA; contact angle $\theta$, 75°) and its mean diameter, porosity, and density are 20 mm, 50 %, and 0.5 g/cm$^3$, respectively. Spore concentrations of *A. sojae* NBRC 32074, *A. oryzae* RIB40, *A. brasiliensis* 6341, *P. griseofulvum* NBRC 7641, *P. chrysogenum* NBRC 4626, and *T. virens* NBRC 6355 were $2.6 \times 10^6$, $3.4 \times 10^6$, $2.6 \times 10^6$, $5.3 \times 10^6$, $5.0 \times 10^6$, and $3.3 \times 10^6$ spores/ml, respectively. The mixture was pored into a PP beaker (diameter, 48 mm; volume, 100 mL) set a hydrophobic polymer net (diameter, 46 mm; thickness, 1 mm; lattice size, 2.5 × 2.5 mm), polyethylene (contact angle $\theta$, 81°) or polypropylene ($\theta$, 91°). Both nets were commercially available for gardening. The net was set up in a microsphere layer (altitude, 10 mm from the bottom of PP beaker). All strains were cultivated at 25 °C by stationary cultivation for 5–10 d. Four duplicates were prepared in each cultivation system.

### 2.3. Construction of agar plate cultivation system set a hydrophobic polymer net

Seventeen mL of PDA diluted by 2 times (agar 1.5 %) was hardened in a PP beaker (diameter, 48 mm; volume, 100 mL). A hydrophobic polymer net, PE, PP, or polytetrafluoroethylene (PTFE; $\theta$, 117°; diameter, 46 mm; thickness, 1 mm; lattice size, 2.5 × 2.5 mm), was set on the surface of the PDA gel. The PTFE net was also commercially available and purchased from AZ ONE Corporation (Osaka). Then, 3 mL of PDA diluted by 2 times was additively pored and hardened onto the net. Thus, the net was buried into a 20 mL of PDA at the depth of approximately 3 mm from the surface on the agar plate. Four hundred μL of spore suspension was spread onto the agar plate (surface area, 18.1 cm$^2$). The concentrations of spore suspensions were as follows: *A. sojae* NBRC 32074, $3.2 \times 10^6$; *A. oryzae* RIB40, $3.6 \times 10^6$; *A. terreus* ATCC 20542, $6.0 \times 10^6$; *P. griseofulvum* NBRC 7641, $1.3 \times 10^6$; *P. multi*color, IAM 7153, $1.3 \times 10^6$; *T. virens* NBRC 6355, $3.1 \times 10^6$. All strains were cultivated at 25 °C by stationary cultivation for 5–10 d. Four duplicates were prepared in each cultivation system.

### 2.4. Analytical methods

The spore production was determined by cell counting method. One cm$^2$ of a fungal mat cut was added into 10 mL of sterilized reverse osmosis water and homogenized for 1 min in an ice bath. After serial dilution of the homogenate with the sterilized water, the spore number was counted with a Thoma's hematometer. All experiments for spore production were performed in four replicates, and the reported results represented as the mean of quadruplicates ± standard derivation. The results were regarded as statistically significant at $p < 0.05$. Residual glucose concentration was determined with Glucose C-II test (Fujifilm Wako Pure Chemical Corporation, Osaka). The residual glucose concentration and final medium pH were measured only about one sample.

## 3. Results and discussion

Concerning the relationship between the physiological and biochemical properties of microbial cells and the hydrophobic environment, some reports have been published so far. Morisaki reported that the increase of respiratory activity and the decrease of glucose uptake of *Escherichia coli* were caused by the adhesion onto some hydrophobic polymers such as styrene–divinylbenzene co-polymer, PTFE, and pyrophylite (Morisaki, 1983). The same phenomenon was observed on a liquid–liquid interface between an aqueous phase and some *n*-alkanes such as *n*-dodecane and *n*-tetradecane (Morisaki, 1984). Moreover, it was also reported that cell division of *Staphylococcus epidermidis* and *Pseudomonas*

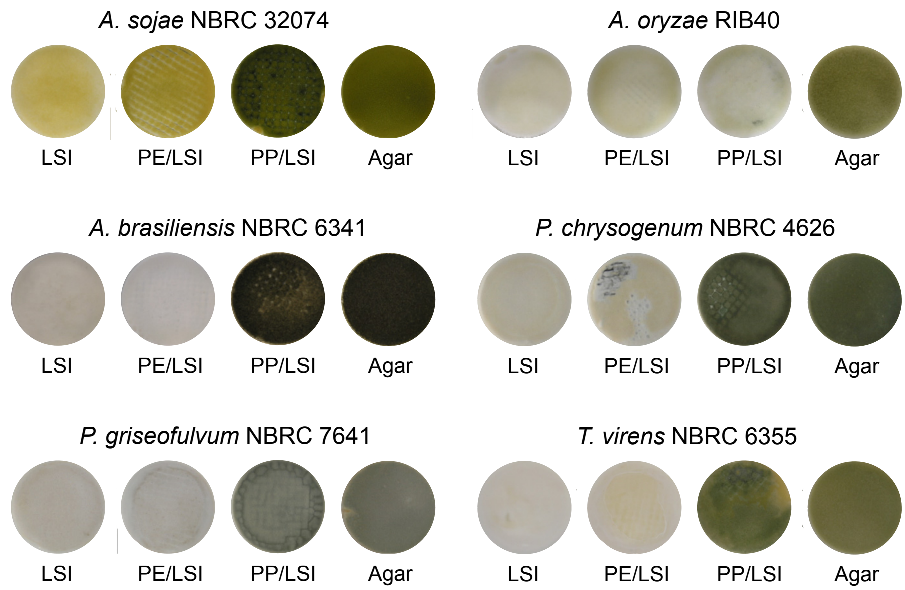


**Fig. 1.** Photographs of fungal mat formed on the surface of LSI, PE/LSI, PP/LSI, and Agar cultivation systems. *A. sojae* NBRC 32074, *A. oryzae* RIB40, *A. brasiliensis* NBRC 6341, and *T. virens* NBRC 6355 were cultivated at 25 °C for 5 d. *P. griseofulvum* NBRC 7641 and *P. chrysogenum* NBRC 4626 were cultivated at 25 °C for 9 and 10 d, respectively.

*aeruginosa* was repressed by the adhesion onto some hydrophobic polymers such as PP, polyethylene terephthalate, and PMMA (Gottenbos et al., 2000).

In addition to bacteria, it was reported that physiological and biochemical properties of yeast cells drastically changed by adhesion onto a hydrophobic solid surface. For instance, pycnidiospores of *Phyllosticta ampelicida* germinated only after adhesion onto a hydrophobic substratum (Shaw and Hoch, 1999), and urediniospores of *Urmyces viciae-fabae* produced extracellular matrices only after adhesion onto a solid surface (Clement et al., 1993). The authors have found that addition of hydrophobic polymer micro-pieces into a microsphere layer of the L–L IBR enhanced subterminal hydroxylation activity of *n*-decane by *Monilliera* sp. NAP 00702 (Oda et al., 2013). Thus, the authors were interested in how the hydrophobic environment affects the fungal spore formation.

### 3.1. Effect of hydrophobic polymer nets in LSI system on spore production

First, effect of embedding a hydrophobic polymer net in a microsphere layer of the LSI system on the spore formation was studied with 6 fungal strains. Four cultivation systems, LSI, LSI embedded a PE net (PE/LSI), LSI embedded a PP net (PP/LSI), and agar plate cultivation (Agar) ones, were prepared in each strain. The

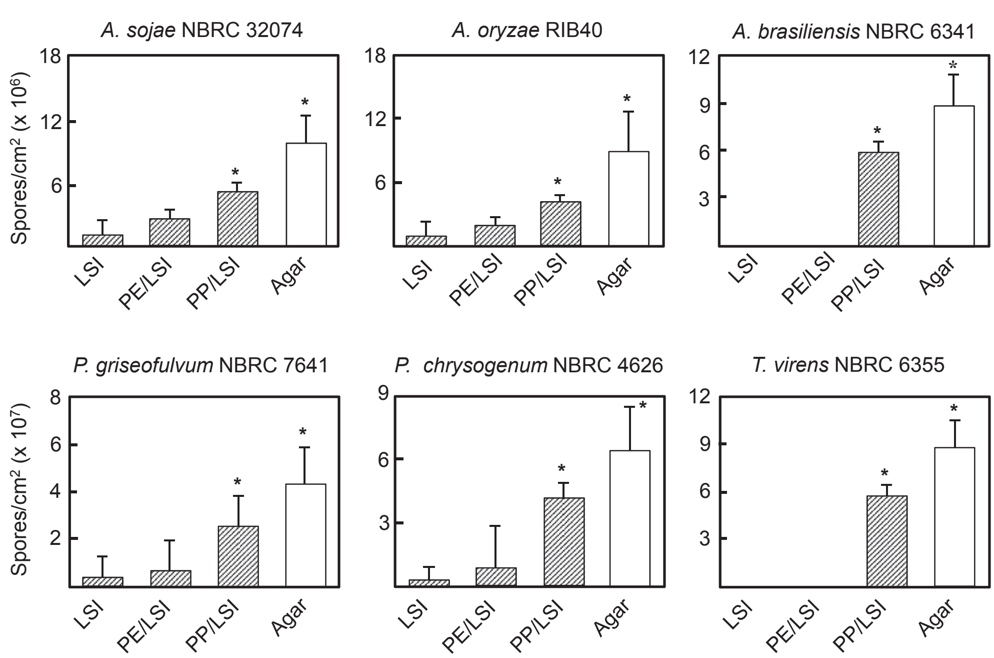


**Fig. 2.** Comparison of spore production among LSI, PE/LSI, PP/LSI, and Agar cultivation systems. After stationary cultivation at 25 °C for 5–10 d, 1 $cm^2$ of a fungal mat was cut off and added into 10 mL of sterilized water. The mixture was homogenized for 1 min in an ice bath, and then spore number in the homogenate diluted serially was counted with a Thoma's hematometer. *Significant differences compared to LSI at $p < 0.05$ in a *t*-test ($n = 4$).

**Table 1**
Final glucose concentration and medium pH in LSI, PE/LSI, and PP/LSI systems.

| | Final glucose concn (g $L^{-1}$) | | | Final medium pH | | |
|---|---|---|---|---|---|---|
| | LSI | PE/LSI | PP/LSI | LSI | PE/LSI | PP/LSI |
| *A. sojae* NBRC 32074 | 9.77 | 8.84 | 5.77 | 5.26 | 5.22 | 3.80 |
| *A. oryzae* RIB40 | 8.14 | 7.06 | 2.85 | 5.16 | 4.59 | 4.53 |
| *A. brasiliensis* NBRC 6341 | 7.53 | 4.82 | 3.22 | 5.86 | 5.29 | 3.63 |
| *P. griseofulvum* NBRC 4626 | 0.74 | 1.46 | n.d. | 4.45 | 4.23 | 3.08 |
| *P. chrysogenum* NBRC 7641 | 0.10 | n.d. | n.d. | 4.47 | 4.07 | 4.20 |
| *T. virens* NBRC 6355 | 8.14 | 7.06 | 2.85 | 5.16 | 4.59 | 4.53 |

n.d., not detected.
*A. sojae* NBRC 32074, *A. oryzae* RIB40, *A. brasiliensis* NBRC 6341, and *T. virens* NBRC 6355 were cultivated at 25 °C for 5 d. *P. griseofulvum* NBRC 7641 and *P. chrysogenum* NBRC 4626 were cultivated at 25 °C for 9 and 10 d, respectively.

LSI system gave the lowest spore production in all strains, especially, *A. brasiliensis* NBRC 6341 and *T. virens* NBRC 6355 hardly produce any spores.

It was reported that the concentration gradients of nutrients developed in an agar plate because diffusion of the sources in the agar plate was substantially limited (Pirt, 1967; Fujikawa, 1994). It is easily assumed that the concentration of carbon and nitrogen sources partially reduced under a fungal mat. It is well known that deficiencies of carbon (Sztejnberg et al., 1990) and nitrogen sources (Krasniewski et al., 2006) are the trigger for production of spores. On the other hand, the carbon and nitrogen sources easily diffuse in an aqueous phase of the LSI system compared with the Agar. Therefore, it is concluded that the agar plate cultivation (Agar) is favorable to the fungal spore production.

However, it was observed that the embedding of a hydrophobic polymer net in a microsphere layer (PMMA; $\theta$, 75°) partially enhanced the spore production in all strains. While the addition of relatively hydrophilic PE net ($\theta$, 81°) barely enhanced spore formation, the addition of more hydrophobic PP net ($\theta$, 91°) significantly accelerated spore production in all strains (Figs. 1 and 2).

Unfortunately, while the growth of all strains in 4 kinds of cultivation systems was not estimated, residual glucose concentration and final medium pH were determined as shown in Table 1. Only one sample in each system was measured. As shown in Table 1, all strains consumed much glucose (initial glucose content, 20.0 g; potato starch content, 4.0 g) and reduced medium pH (initial value, 6.0). Moreover, it was also observed that all strains tested formed a physically strong fungal mat with microspheres on the surface of a PDA broth. All PP/LSI systems producing a lot of spores exhibited much glucose consumption compared with LSI and PE/LSI.

### 3.2. Effect of hydrophobic polymer nets in an agar plate on spore production

As mentioned above, although the LSI system is unfavorable to produce fungal spores compared with the agar plate cultivation, it was observed that the embedding a PP net in a microsphere layer accelerated spore formation (Figs. 1 and 2). The effect of a PE net was insufficient because of its low hydrophobicity. Thus, it was expected that the contact of vegetative hyphae with a hydrophobic polymer net embedded in an agar plate enhanced the spore formation.

As shown in Figs. 3 and 4, it was observed that the spore production by *A. oryzae* RIB40, *A. terreus* ATCC 20542, *P. griseofulvum* NBRC 7641, and *P. multicolor* IAM 7153 was enhanced by setting a PP or a PTFE ($\theta$, 117°) net in an agar plate. Especially, embedding of a PTFE net (PTFE/Agar) significantly enhanced the spore formation by *A. oryzae* RIB40, *A. terreus* ATCC 20542, and *P. multicolor* IAM 7153

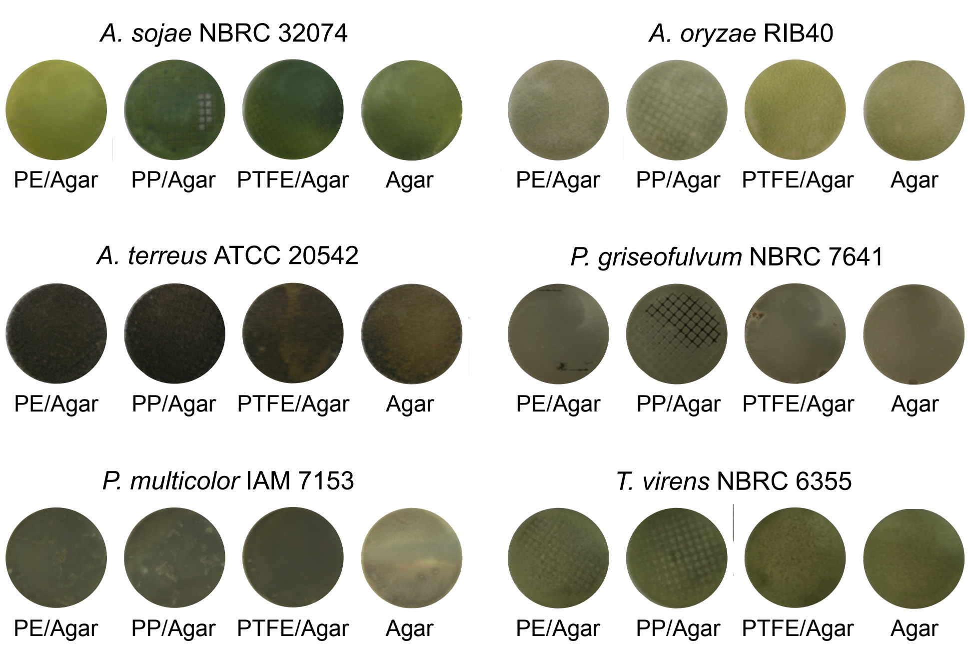


**Fig. 3.** Photographs of fungal mat formed on the surface of PE/Agar, PP/Agar, PTFE/Agar, and Agar cultivation systems. *A. sojae* NBRC 32074, *A. oryzae* RIB40, *A. terreus* ATCC 20542, and *P. multicolor* IAM 7153 were cultivated at 25 °C for 5 d. *P. griseofulvum* NBRC 7641 and *T. virens* NBRC 6355 were cultivated at 25 °C for 7 and 9 d, respectively.

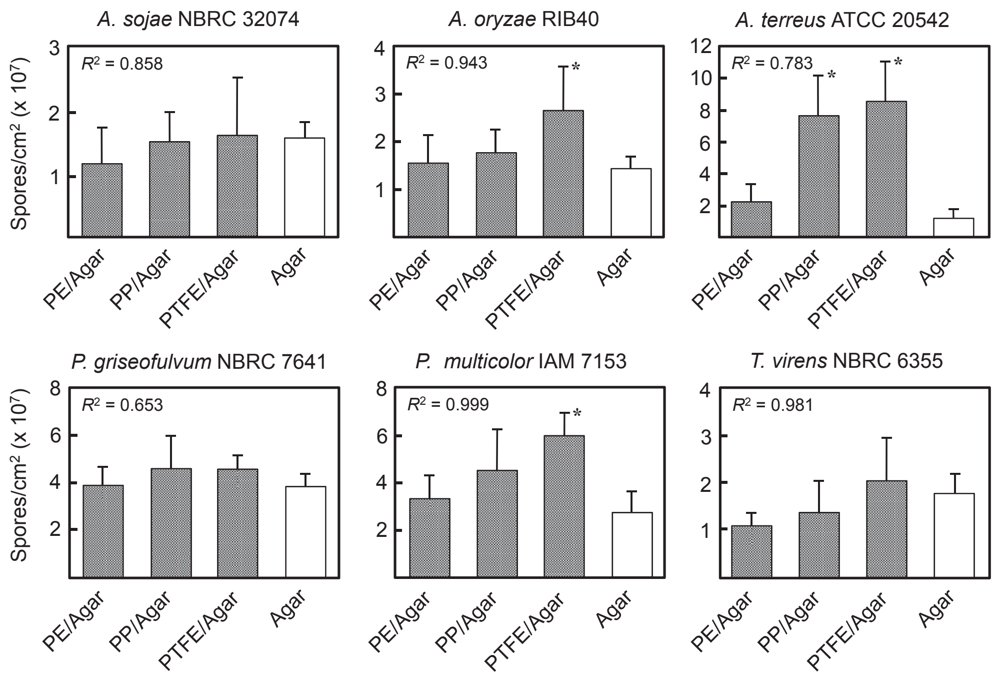


**Fig. 4.** Comparison of spore production among PE/Agar, PP/Agar, PTFE/Agar, and Agar cultivation systems. After stationary cultivation at 25 °C for 5–9 d, 1 $cm^2$ of a fungal mat was cut off and added into 10 mL of sterilized water. The mixture was homogenized for 1 min in an ice bath, and then spore number in the homogenate diluted serially was counted with a Thoma's hematometer. *Significant differences compared to Agar at $p < 0.05$ in a $t$-test ($n = 4$). The coefficient of determination ($R^2$) was calculated between the hydrophobicity of net and the spore production.

compared with that in conventional agar plate cultivation (Agar). Positive correlations between the hydrophobicity of net and the spore production were observed in all strains. The coefficient of determination ($R^2$) of the positive correlation ranged from 0.653 to 0.999 as shown in Fig. 4. Thus, it is concluded that the embedding of a hydrophobic polymer net such as PTFE in an agar plate is effective and easy for the enhancement of spore production of many fungi.

Although the mechanism for the enhancement of spore production by hydrophobic polymer net is unfortunately unknown as of now, the authors assume that hydrophobic environment plays as some physical stimulant factors affecting the characteristics of microbial cells besides above-cited some literature.

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