# Culture media for growth of mycelium and for induction of sporulation of *Ascosphaera apis*, the causative agent of *chalkbrood disease of honey bee (Apis mellifera L.)*

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## Abstract

An investigation of media suitable for growth and development of mycelium of the fungus, *Ascosphaera apis*, the causative agent of chalkbrood disease (CBD) in honey bee, *Apis mellifera* L, was carried out. Five culture media: Sabouraud dextrose agar + 0.2% Yeast extract (SDYEA), Malt yeast extract + 20% dextrose agar (MY 20), Jasmine brown steam rice (JBSR) and SDYEA + 5% bee hemolymph (SDYEAH) were used to demonstrate the fungal growth compared with that of culture on the standard medium, potato dextrose agar (PDA). Diameter of the mycelium colonies were compared. Results showed that among the tested media the diameters of mycelium colonies were significantly different ($P \leq 0.05$). The sizes of mycelium colonies on MY20 were the largest, followed by those on SDYEAH, SDYEA, PDA and JBSR, respectively. The thicknest hyphae or highest biomass was found on the SDYEAH, followed by MY20, SDYEA, PDA and JBSR, respectively. However, JBSR could prolong the hyphal longevity through 90 days without subculturing. Medium that suitable for induction of sporulation was MY20. Amount of spore produced on MY20 was more than those from malt yeast extract + 30% dextrose agar (MY30) and SDA, but size of ascocarp and ascus in MY30 was bigger than those on MY20 and SDA significantly ($P \leq 0.05$), however size of ascospores were significantly different ($P \leq 0.05$).

**Keywords:** *Ascosphaera apis,* chalkbrood, honey bee, culture media, induction of sporulation.

## Introduction

Chalkbrood disease is honey bee epidemic cause by *A. apis*. After infection, the fungal hyphae grow to cover the bee larvae which is then called mummified larvae. The fungal can rapidly spread to other bee larvae, bring the bee cultivation fails. For studying the biology and pathogenicity of *A. apis*, the culture has to be maintained by transfer to fresh growth media.

By using standard media for fungus such as Potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA), is necessary to subculture every two weeks which is time consuming. Raffinengo, et al[1] found that integral rice kernel (IRK) prolonged the fungal hyphal longevity through 60 days without subculturing whereas Rosalind and Buckner[2] found that plant oil and bee larvae lipid increased spore germination from 50% to 70-80%.

In this study *A. apis* was grown on different growth media, some were modified from standard media. The ability, prolong mycelia longevity and the ability to induce mycelia develop to be ascospore of these media were compared.

## Experimental

### Material

Take samples (mummified larvae) of *A. apis* from Petchabun province (*A. apis*, Petchabun isolate) was chosen in this research.

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## Method

**Material for resting the ability to prolong mycelial longevity.**

Potato agar (PDA) was used as standard media to compare with other 4 media: Sabouraud dextrose agar + 0.2% yeast extract (SDYEA), Malt yeast extract agar + 20% dextrose (MY20), Jasmine Brown Steam Rice (JBSR) and SDYEA +5% Bee hemolymp, H (SDYEHA).

The mummified larvae soak in sterile distilled water for 5 min to remove the dirt and wipe to dry then cut it to small pieces and place on the prepared media one piece on each plate. Incubate at 30±5°C for three days, 0.5 cm. diameter cork borer was used to bore at the colony edges. A bored piece was taken to place at the middle of new media plate. (Five plates for each type of media.) Incubate at 30±5°C and record the fungal colony diameter, and mycelia density. The difference between media was analyzed by using ANOVA.

**The media testing the ability to induce mycelial develop to ascospore**.

Three types of media which different in amount of carbon source (glucose) were
investigated for suitable for sporulation: Malt yeast extract agar + 20% dextrose (MY20), Malt yeast extract agar + 30 % Dextrose (MY30) and Sabouraud dextrose agar (SDA) was standard media.

The 0.5 cm diameter cork borer was used to cut at colony edge to place on new media plate. Incubate at 30±5°C for more than 10 days. In each plate, cut the sporadic area three pieces sequencing from the center of the plate by using cork borer. Scrape the agar surface containing spores and put in 15 ml. centrifugal tube, filling 2 ml. sterile distilled water were plus 0.01% triton X-100. Count the ascocarp amount by haemocytometer. The size of ascocarp, ascus and ascospore were determined on cork borer disk by staining with lacto phenol cotton blue under light microscope. Each media were experimented five replicates. The difference between media was analyzed using ANOVA.

## Results and Discussion

**The media testing the ability in prolong mycelia longevity**

The test for ability in prolonging mycelia longevity was done by cutting agar at the edge of 3-day fungal colony and placed on five types of media SDYEA, MY20, JBSR, PDA and SDYEHA, after incubation at 30±5°C, the colonies' diameters were measured for 7 days. It was found that the mycelia of *A. apis* Petchabun isolate most rapidly grew on media MY20 with the largest colony, following by SDYEHA and SDYEA with comparably grew and PDA, JBSR respectively. (Table 1)

**Table 1** Diameter of *Ascosphaera apis*, Petchabun isolate colonies, on five types of agar in day 1 to day 7.

| ***Media*** | ***Colony diameter of Ascosphaera apis (cm.)*** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Day1** | **Day2** | **Da3** | **Day4** | **Day5** | **Day6** | **Day7** |
| PDA | 0.694[a] | 1.69[a] | 2.754[b] | 3.858[b] | 4.868[b] | 5.472[b] | 6.364[b] |
| | ±0.043 | ±0.259 | ±0.289 | ±0.476 | ±0.533 | ±0.546 | ±0.611 |
| SDYEA | 1.184[c] | 3.292[c] | 4.934[c] | 6.374[c] | 7.352[c] | 8.082[c] | 8.5[c] |
| | ±0.105 | ±0.139 | ±0.173 | ±0.175 | ±0.241 | ±0.050 | ± 0.000 |
| SDYEAH | 1.444[d] | 3.318[c] | 5.124[c] | 6.544[c] | 7.684[c] | 8.5[c] | 8.5[c] |
| | ±0.043 | ±0.189 | ±0.114 | ±0.257 | ±0.196 | ±0.000 | ±0.000 |
| JBSR | 0.860[b] | 1.136[b] | 1.36[a] | 2.212[a] | 3.104[a] | 3.824[a] | 5.026[a] |
| | ±0.151 | ±0.109 | ±0.050 | ±0.075 | ±0.082 | ±0.283 | ±0.842 |
| MY20 | 1.464[d] | 4.162[d] | 6.53[d] | 8.092[d] | 8.336[d] | 8.368[c] | 8.5[c] |
| | ±0.065 | ±0.169 | ±0.346 | ±0.400 | ±0.233 | ±0.197 | ±0.000 |

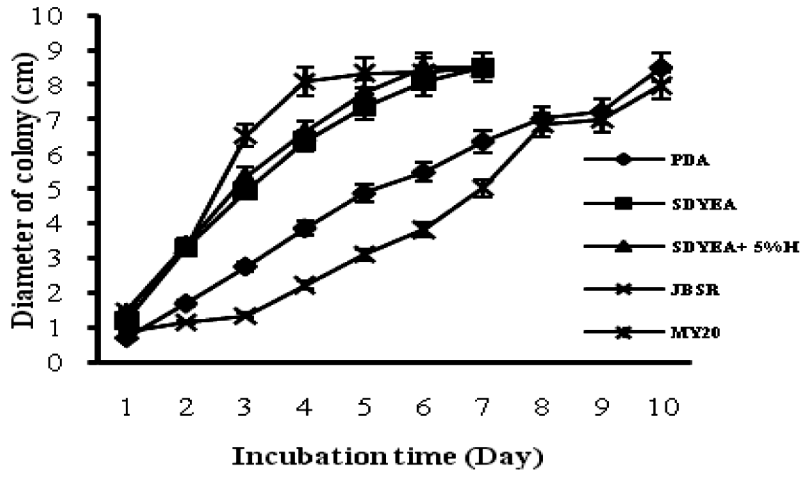


**Figure 1** The Ascosphaera apis, Petchabun isolate mycelia growth on 8.5 cm. plate. The colonies' diameter' were measured for 10 days on JBSR and PDA. 7 days measurement on SDYEA, MY20 and SDYEHA due to full mycelial growth on plate.

The growth increased each day from day 1 to day 10, according to figure 1. After culturing for 3 and 5 days on the media SDYEHA, the mycelia was more dense and fluffy than other media. (Table 2)

**Table 2** Ascosphaera apis, Petchbool isolate mycelial fluffiness on five type of media agar on day 3 and day 5.

| ***Media*** | ***Fungal fluffiness of Ascosphaera apis*** | |
|---|---|---|
| | ***Day3*** | ***Day5*** |
| Sabouraud dextrose + yeast agar (SDYEA) | 2 | 2 |
| Sabouraud dextrose + yeast agar + hemolymph (SDYEHA) | 3 | 3 |
| Malt yeast extract agar (MY 20) | 2 | 2 |
| Potato dextrose agar(PDA) | 1 | 1 |
| Jasmine brown stream rice (JBSR) | 1 | 1 |

**Table 3** Ascospore densities of Ascosphaera apis, Petcahbun isolate on bored agar of three types of media.

| ***Media*** | ***Amount of ascospore(spores/ml)*** |
|---|---|
| Sabouraud dextrose agar (SDA) | $1.55^{a} \times 10^{6} \pm 0.716$ |
| Malt yeast extract agar (MY 20) | $3.20^{c} \times 10^{6} \pm 0.647$ |
| Malt yeast extract agar (MY 30) | $2.45^{b} \times 10^{6} \pm 0.647$ |

**Table 4** Size of ascocarp, ascus and ascospore of Ascosphaera apis Petchabun isolate on three types of media.

| *Media* | *Size of ascocarp, ascus and ascospore (μm)* | | |
|---|---|---|---|
| | **ascocarp** | **ascus** | **ascospore** (wide x length) |
| Sabouraud dextrose agar (SDA) | 70.7 [a]±2.171 (62.5 - 80) | 12.8 [a]±1.333 (7.5 -17.5) | 2.2 [a]±0.196 x 5.1[a]±0.048 |
| Malt yeast extract agar (MY 20) | 82.9 [b]±3.273 (67.5 - 92.5) | 14.1 [b]±1.080 ( 10 -17.5 ) | 2.4 [b]±0.116 x 5.3 [b]±0.082 |
| Malt yeast extract agar (MY 30) | 90.1 [c]±2.908 (75 -102.5) | 17.4 [c]±1.398 ( 10 - 20) | 2.5 [c]±0.116 x 5.3 [b]±0.164 |

This may be due to the unnecessity of the mycelia for adaptation to the new media. The reason was the media had the composition of hemolymph, which was corresponded to the research of Kimbrough and Atkinson[3].

The fungal *Hymenoscyphus caudatus* could grow and sporulated well on the imitated media composed of host tissue, however, the concentration percentage of hemolymph had no different effect, with significance at $P \geq 0.05$, on the growth of mycelia on solid media.

**The media testing the ability to induce mycelia develop to be ascospore.**

**Spore density.**

The area spore grew was cut from the center of the plate sequence for three pieces in one plate by using 0.5 cm. diameter cork borer. The spore area on the agar was scraped and put in 15 ml. centrifugal tubes, filling with 2 ml. sterile distrilled water were plus 0.01% tritonX-100.

Ascospore amounts were counted by haemocytometer under light microscope The ascospore number on media MY20 was more than MY30 and SDA; 3.20 x $10^6$, 2.45 x $10^6$ and 1.55 x $10^6$ spore/ml. respectively, which differed significantly with $P \leq 0.05$ (Table 3). The result was corresponded to those of Wu and Youssef[4]. They found the media with glucose resulted in more spore formation than the media with ammonium sulfate more than 0.25% or without sugar. However, in media MY30, the ascospore amount of *A. apis* Petchabun isolate was 2.45x$10^6$ spore/ml, which was less than media MY20, but higher than media SDA. This may be due to too high carbon source (more than 20%), which unsuitable for sporulation.

**Ascocarp, ascus and ascospore sizes.**

The average size of ascocarp, ascus and ascospore on three types of media were measured.

Ascocarp were found to different significantly at $P \leq 0.05$. However, ascus sizes were different significantly at $P \leq 0.05$ (Table 4). On media MY30, the ascocarp sizes were the biggest, ascus sizes were comparable and the average sizes were 90.1 μm. and 17.4 μm., respectively. On media MY20, the average sizes were 82.9 μm. and 14.1 μm., respectively. On SDA media, the average sizes were 70.7 μm. and 12.8 μm., respectively. The results were corresponded to that of Ruffinengo, et al[1]. They found *A. apis* cultured on MY2 with glucose only 2%. Ascospore which had the figure of oval-long shape had comparable which on SDA, MY20 and MY30, were different significantly at $P \leq 0.05$ (Table 4). On media MY30, the average size was 2.5 x 5.3 μm. on MY20 was 2.5 x 5.3 μm. and on SDA was 2.2 x 5.1 μm. thus differed from on MY20 and MY30 significantly at $P \leq 0.05$, but between on MY30 and MY20 were not different significantly at $P \geq 0.05$ in length.

## Conclusion

Suitable media for complete mycerial growth were SDYEHA, MY20 and SDYEA. The media for the

most rapid mycelia growth was MY20. The media which mycelia grew the most dense was SDYEHA.

The media able to the most prolong mycelia longevity more than 90 days was JBSR without subculture. This made the media suitable for genetic maintenance.

The media suitable for sporulation induction was MY20 and the media producing the largest ascocarp and ascus was MY30.

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