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Evaluation of Substrates for Mass Culture of *Phaeoacremonium parasiticum*, the Fungus Responsible for Artificial Oleoresin Deposition in Agarwood Plant

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Abstract

For establishing the favourable media for growth of *Phaeoacremonium parasiticum*, the fungus was inoculated in five different media (Potato Dextrose Broth (PDB), Host Extract (50%) + PDB (50%) (HE + PDB), and the solid media *viz*., Rice Bran (RB), Maize Meal (MM) and Wheat Bran (WB)) at pH of 6.5 and incubated at a temperature of 30˚C ± 1˚C. The solid media were added with water at the rate of 70 ml/100g of solid substrate. After 30 days of inoculation, conidial population of *P. parasiticum* in liquid media was lower compared to conidial population in solid media. In solid media, MM media showed the highest conidial population (9.56 log·cfu/g) followed by WB (9.50 log·cfu/g) and RB (9.38 log·cfu/g). Hence, Maize Meal media (MM: Water = 100 g:70 ml) at pH of 6.5, incubation temperature: 30˚C ± 1˚C for 30 days would be the standard technique for mass production of *P. parasiticum*.

Keywords

Phaeoacremonium parasiticum, Agarwood, Standardization, Mass Culture, Media

1. Introduction

Agarwood, also known as *Uud, Oodh, Agar or Gaharu*, is the dark resinous heartwood that forms in *Aquilaria* and *Gyrinops* trees known as Agar trees. Until recently, Agarwood was primarily produced from *Aquilaria malaccensis* [1] for three principal uses like medicine, perfume and incense. The international trade in Agarwood involves wood, wood chips, powder and oil [2]. It is used in mos-

ques and burned to honor guests as well as burned in preparation for prayer [3]. More modern studies confirmed that, Agarwood has bioactive products that function as effective anti-microbial compounds. Agarwood may have anticancer activity, and can be used as an antidepressant and used to promote good health [4] [5]. The global perfume industry has become interested product, and Agarwood has also caught the interest of esoteric circles of non-Asian societies. Agarwood has become the most expensive perfumery raw material in the world and its value surpasses the value of gold [6]. The price of Agarwood is from few dollars per kilogram to over 30,000 US dollars for top quality resinous wood in international market [7]. Seeking and cutting down the tree for harvesting Agarwood has made the Agar trees in nature reduced. To meet the demand of supplying to the market, lots of Agar plants have been grown from small scale at farmer house to large scale in farm.

Even they have highly useful and highly economic value, but unfortunately, in natural forests, the Agar tree only produces oleoresin when it is infected by fungus and only 7% - 10% of the trees are infected by the fungus [8]. The other report showed that in fact, only 1% of Agar trees in nature contain the resinous Agarwood substance [9]. Currently, various researches and investigations concluded that the oleoresin forming in Agar tree relates to the certain contaminated factors combine with certain conditions [2]. The resin production is response to fungal infection and *Phaeoacremonium parasiticum* is one of the known fungi reported to be associated with Agarwood formation [1] [10] [11]. Its pathogenesis was confirmed and the technique for its isolation and *in-vitro* culture method has been developed. The result also showed that conidial population density of *P. parasiticum* in Potato Dextrose Broth was 6.24 log·spore/ml and material used for mass culture was costly [12]. Hence, study of evaluation of substrates for mass culture of *Phaeoacremonium parasiticum* was done to achieve the maximum potential sporulation of *P. parasiticum* with acceptable cost.

2. Materials and Methods

2.1. Evaluation of Media for Mass Culture of *P. parasiticum*

The media included the liquid media *viz.,* Potato Dextrose Broth (PDB), Host Extract (50%) + Potato Dextrose Broth (50%) (HE + PDB), and the solid media *viz.,* Rice Bran (RB), Maize Meal (MM), Wheat Bran (WB). The solid media were added with water at the rate of 70 ml/100 g of solid substrate. pH was adjusted at 6.5 by adding HCl or NaOH. Mycelial disc of 7 mm diameter size was cut with the help of cork-borer from 30 days old of *P. parasiticum.* Substrates were inoculated with freshly cultured mycelial disc and inoculated media were incubated in BOD incubator (ICT) at 30˚C ± 1˚C. The treatments were replicated 5 times. The log·cfu/g or log·spore/ml was counted after 30 days of inoculation by pour plate method in PDA plates, to identify the media in which the fungus have the highest inoculum. Haemocytometer was used to count the

log·spore/ml of liquid media.

2.2. Calculation of Colony Forming Unit (CFU)

The colony forming units per petriplate were counted with the help of the marker (25 petriplates were counted per treatment). Number of colony forming unit per g or ml of sample was calculated follow the formulation [13] as below and converted log values.

Number of colony forming unit/g or ml of sample = (Number of cfu/plate) × (reciprocal of dilution of sample).

3. Results and Discussion

Phaeoacremonium parasiticum in MM and WB media grew faster and covered all the media in conical flask after 22 days of inoculation (**Figure 1(b)** and **Figure 1(c)**). The back side of conical flask of MM media changed to black associated with white colour (**Figure 1(b)**) and colour of the back side of conical flask of WB media was white (**Figure 1(b)**). In RB media, at 30 days of inoculation, growth of fungus could not cover full the medial surface (**Figure 1(a)**). Growth of *P. parasiticum* was very slow in liquid media compared to solid media (**Figure 1(d)** and **Figure 1(e)**).

Ajello *et al.* [14] showed cardinal temperatures for *P. parasiticum* growth as 15˚C (minimum), 25˚C (optimum), and 35˚C (maximum). The other study suggested that discrepant optimal growth temperatures have been obtained for *P. parasiticum*, ranging from 25˚C - 30˚C and the optimum was closer to 30˚ than to 25˚C [15]. Akhila *et al.* [12], after trying incubating *P. parasiticum* at temperature *viz.*, 25˚C ± 1˚C, 27˚C ± 1˚C, 30˚C ± 1˚C and 32˚C ± 1˚C in PDA media, concluded that optimal growth temperature of *P. parasiticum* was 30˚C ± 1˚C. Also in the same study, the optimum growth pH of *P. parasiticum* was suggested at 6.5 after trying at pH *viz.*, 5.0, 5.5, 6.0, 6.5 and 7.0. Based on the previous studies, the temperature for growth of *P. parasiticum* was set at 30˚C ± 1˚C and pH was set at 6.5 for the present study.

The result of population density (cfu/g or ml) count showed that, in liquid media, PDB supported the highest growth with 7.23 log·cfu/ml followed by HE + PDB with 4.85 log·cfu/ml. MM media as solid media supported for maximum conidial population (9.56 log·cfu/g) of the fungus, followed by WB (9.50 log·cfu/g) and RB (9.38 log·cfu/g) (**Table 1** and **Figures 2(a)-(d)**). The result of liquid media (PDB and HE + PDB) observed in the present study is in accordance with the study by Akhila *et al.* [12]. They also tried mass culture of *P. parasiticum* in liquid media, and after 20 days of inoculation, the spore population in PDB was higher than the spore population in HE + PDB. Zhang *et al.* [16] reported that the Host Extract (HE) from the healthy part of Agar tree contains a low amount of Agawood oil compound as a defence agent against *P. parasiticum.* Hence, in the HE media or in the added HE media, the *P. parasiticum* growth was lower compared to the growth in other media. This result is in agreement

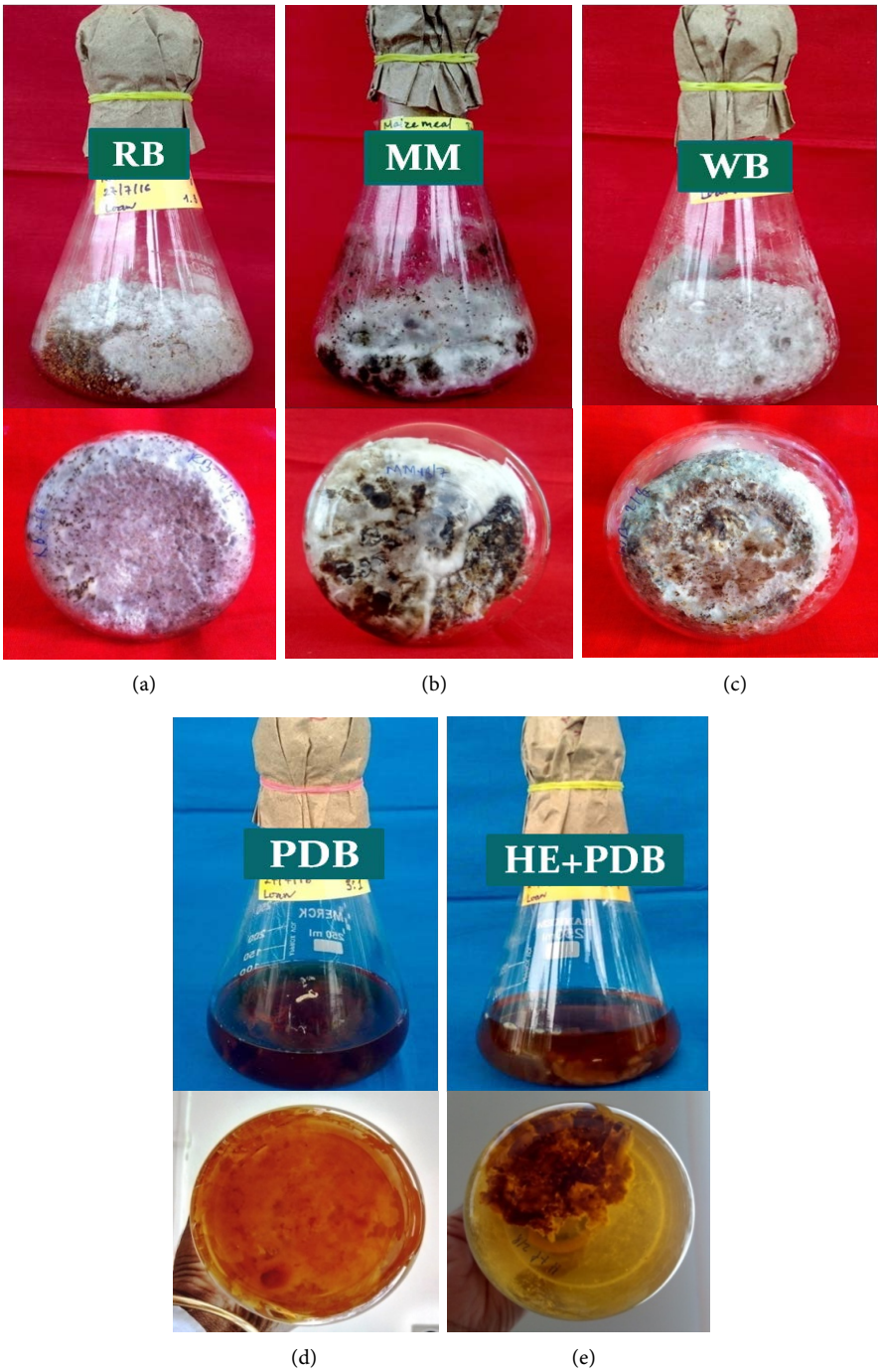


Figure 1. (a)-(d) Growth of *P. parasiticum*in different mass culture after 30 days of inoculation. (a) Rice bran; (b) Maize meal; (c) Wheat bran; (d) Potato dextrose broth; (e) Host extract (50%) + PDB (50%).

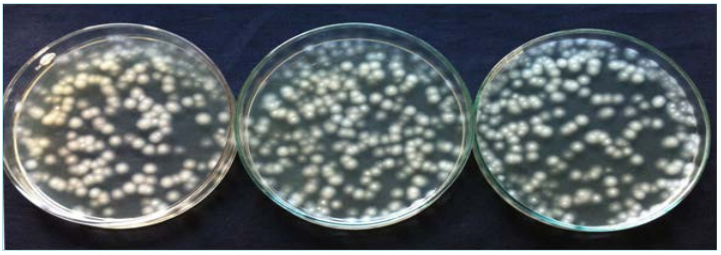
with various studies which stated that, the Agarwood extract originating from Agar trees exhibited strong antifungal activity against *Fusariumsolani, F. oxysporum, Candida albicans, Lasiodiplodia theobromae* [16] [17] [18] [19].

Mass culture of *Beauveria bassiana* was done by the several workers. According to each study, the best result of spore production in different media was

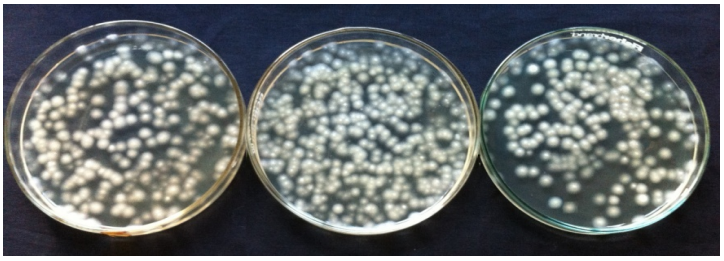
Table 1. Population density of *P. parasiticum* in different culture media after 30 days of inoculation.

Treatment	No. of cfu/g or ml*	Log cfu/g or ml**
T_1: Rice Bran	243×10^7	9.38^c
T_2: Maize Meal	367×10^7	9.56^a
T_3: Potato Dextrose Broth (PDB)	17×10^6	7.23^d
T_4: Wheat Bran	316×10^7	9.50^{ab}
T_5: HE (50%) + PDB (50%)	72×10^3	4.85^e
SEd (±)		0.0571
$CD_{0.05}$		0.1273

*Mean of 25 replications; HE: Host Extract; **Mean within the same column followed by the same letter are not significant different at 0.05 level of probability.



(a)



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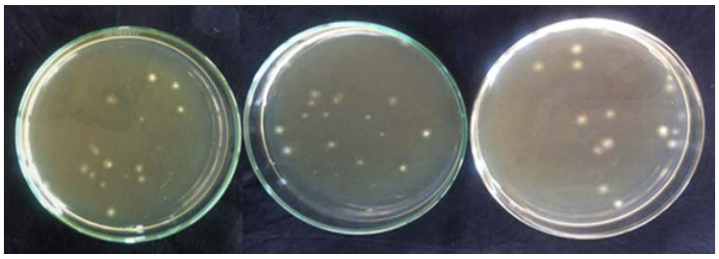

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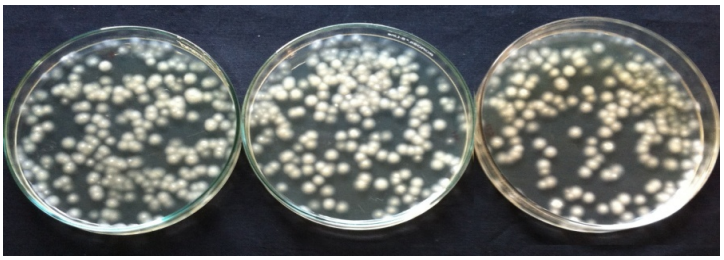
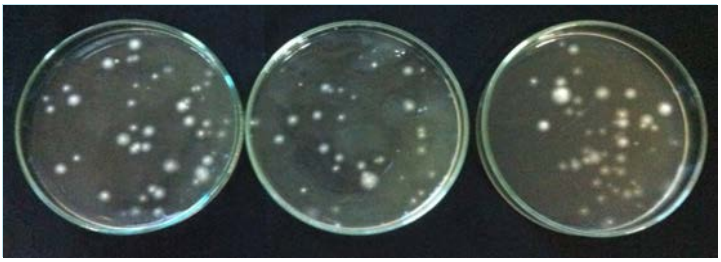


(d)



(e)

Figure 2. (a)-(e) Population density (cfu/g or ml) of *P. parasiticum* in different mass culture media. (a) Rice bran (10^{-7} dilution); (b) Maize meal (10^{-7} dilution); (c) Potato dextrose broth (10^{-6} dilution); (d) Wheat bran (10^{-7} dilution); (e) Host extract (50%) + potato dextrose broth (50%) (10^{-3} dilution).

showed as: in Rice flour liquid media (2.4×10^8 spores/ml), and in Rice media (6.24×10^4 spores/g) [20], in Cowpea solid media (9.06×10^7 spores/g) [21]. Mass culture of *Nomuraea rileyi* showed the best spore production in Rice solid media (5.53×10^7 spore/g) and higher spore production in Wheat solid media (3.55×10^7 spores/g) [22]. In mass culture of *Trichoderma viride,* the highest conidial quantity assessment was in Potato Dextrose Agar (91.89×10^8 cfu/ml) [23].

In mass culture production of *Deuteromycetes* fungi, solid media *viz.,* Rice Bran (RB), Maize Meal (MM), Wheat Bran (WB) always are considered to be used for study because they are the common products, available, relatively cheaper and supporting both mycelia growth and spore production. Among those, Rice or Rice Bran usually gives the better result [24] [25] [26]. But in the present study, for mass culture of *P. parasiticum*, MM showed as the best substrate followed by WB. Maize contains high proportions of carbohydrates (starch), and to a lesser extent minerals, fats and proteins [27]. Lancey [28] stated that, because the whole maize is a good source of thiamin, pyridoxine and phosphorus, and a fair source of riboflavin, niacin, folate, biotin, iron and zinc, so maize meal is one of the favourite substrate for fungi development that many cause nutritional losses and production of toxic substances known as mycotoxin. According to Pollack and Benham [29], Maize meal was a well-established mycological medium used for the cultivation of fungi and to study chlamydospores production of various fungi.

Wheat Bran, a by-product of the dry milling of common Wheat, is one of the major agro-industrial by-products used in animal feeding. It consists of the outer layers (cuticle, pericarp and seedcoat) with small amount of starchy endosperm of the Wheat kernel. Wheat Bran has been used in fungal culture in laboratory as well as fungal mass culture in industrial scale [30].

The present study showed that, Maize Meal media (MM: Water = 100 g:70 ml) at pH of 6.5, incubation temperature: 30˚C ± 1˚C for 30 days would be the standard technique for mass production of *P. parasiticum.*

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