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Apple pomace as effective substrate for growth and spore production of entomopathogenic fungi, *Lecanicillium lecanii*, *Beauveria bassiana* and *Paecilomyces fumosoroseus*

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Apple pomace (AP), the left over waste after extraction of juice, is often dumped in open field and that adds to environmental pollution. In this context and a rich source of carbohydrates, we tried to standardize the AP as a substrate for growth and spore production of entomopathogenic fungi (EPF) viz., *Lecanicillium lecanii*, *Beauveria bassiana* and *Paecilomyces fumosoroseus* by adding water, ammonium nitrate, as well as using different temperatures and pH. Results have shown that addition of 40 mL of water, 4 g of ammonium nitrate, and maintaining temperature at 30°C and pH alkaline (pH 8 & 10) in AP recorded significantly higher spore production of *L. lecanii* (50.53, 52.81, 151.2 and 50.26-52.2 lakh spores/mL, respectively), *B. bassiana* (50.44, 51.87, 152.2 and 50.14-51.66 spores/mL, respectively) and *P. fumosoroseus* (50.56, 52.18, 149.3 and 50.14-52.31 lakh spores/mL, respectively) as compared to positive control, potato dextrose agar (41.76-43.8 lakh spores/mL).

Keywords: Ammonium nitrate, Apple waste, *Mallus domestica*

Apple, *Mallus domestica* Borkh is the most important fruit widely grown in temperate countries. The area of apple crop in the world is about 49.34 lakh hectares with a production of 8.31 crore tonnes during 2017[1]. The total annual production of apples in India is about 2327 metric tonnes with an area of 301 thousand hectares during 2017-18. Out of total production, 71% of the apple fruits are used for consumption, 20% is for value added products. Apple pomace (AP) is a left-over waste or by-product after extraction of juice contains peel and seeds which represents about 25 to 35% of weight of processed apples[2]. Even when the total annual production of AP in India was about 1.3 million in 2010, only 10000 tonnes were utilized and the remaining was dumped in open fields creating environmental pollution[3]. AP contains (in dry wt. basis) 3.97-5.4% moisture, 48-62% carbohydrates (glucose 22.7%, fructose 23.6%, sucrose 1.8%), 57.85% total soluble solids, 4.45-5.67% proteins, 3.29% soluble proteins, 8.53-18.50 mg/100 g vitamin C, 4.7-51.1% fibre (insoluble fibre 36.5%, soluble fibre 14.6%) 3.90 pH, 1.52% amino acids, 3.5-14.32% pectin and 0.99% polyphenol[2].

In apple fruit processing industries, disposal of apple waste (pomace) generated during pre- and post-processing of apple fruits is a potential challenge. Besides polluting environment, it poses health hazards due to growth of undesirable microbes. However, at present, AP is being utilized for production of value added products viz., ethanol, crude protein, pectin, microbial colours, citric acid, dietary fibre and animal feed[2,3]. Further, to add to effective utilization of this biowaste, we studied if it could be used as a substrate for entomopathogenic fungi.

Entomopathogenic fungi (EPF) are used as biological control agents in the management of insect pests (sucking pests, chewing insects, nematodes) and powdery mildew disease in agriculture[4]. Food grains such as rice, wheat, bajra, etc., are generally used as a solid substrate for mass production of EPF. In India, biopesticide industries are using liquid media (sugarcane molasses yeast broth) for commercial production of EPF blastopore's due to non-availability of cost effective solid agro wastes. Development of cost-effective mass production technology is a prerequisite for EPF. Apple is the major fruit crop in Himachal Pradesh, Jammu & Kashmir and Uttarakhand states of India.

Any fungi require rich source of carbohydrates, nitrogen and neutral to alkaline pH for better growth and multiplication. AP is highly acidic (pH 3.7-3.9), rich in carbohydrates but lacks nitrogen. Therefore, it is necessary to supplement the AP with nitrogen and to modify the pH for better growth and multiplication of EPF. Hence, in the present study we explored the potential of apple pomace (AP) as a substrate for optimal mass production of entomopathogenic fungi *Lecanicillium lecanii*, *Beauveria bassiana* and *Paecilomyces fumosoroseus* in terms of growth and spore production by adding water, ammonium nitrate, and monitoring the temperature and pH.

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Materials and Methods

Production of apple pomace powder

Fresh AP collected from Fruit Processing Plant, Himachal Pradesh Marketing Co-operative Society (HPMC), Parwanoo, Govt. of Himachal Pradesh was dried under shade for 10-15 days and was powdered using grinder.

Maintenance of EPF cultures

Pure cultures of *L. lecanii*, *B. bassiana* and *P. fumosoroseus* was obtained from the ICAR-National Bureau of Agricultural Insect Resources (NBAIR), Bangalore and multiplied on potato dextrose agar (PDA) as and when required for experiments.

Effect of various factors in AP powder on growth and spore production of EPF

Water

Ten grams of AP powder was taken in 250 mL conical flasks; 0.5 g of calcium carbonate was added in different concentrations of water (15, 20, 25, 30, 35 and 40 mL) separately. The mixture was mixed properly using glass rod and kept for one hour. All flasks were autoclaved at 121°C for 20 min and kept at room temperature for 24 h to check contamination, then inoculated with 1.0 mL spore suspension of EPF under aseptic conditions. The culture flasks were incubated at 27°C for 10 days and then the spore yield (no. of spores/mL) was calculated after harvesting the mycelia. There were six treatments and each treatment replicated thrice.

Harvesting of EPF and counting the spore production

Entomopathogenic fungi were allowed to multiply in the AP medium up to 10 days in 250 mL conical flasks. Tween 80 (0.25%) solution was prepared with distilled water and 100 mL was added to each flask and allowed to soak for 1 hour. The AP media containing conidia/mycelia was filtered with double layered muslin cloth to separate the mycelium. The filtrate was transferred to 100 mL centrifuge tube and allowed for centrifugation at 2000 rpm for 5 min at 4°C. The supernatant was discarded and the sediment was mixed with 10 mL of distilled water and then number of spores/mL was counted under stereo-binocular microscope using Neuber Haemocytometer, then spore yield/mL was calculated using the following formula.

$$Number\ of \frac{spore}{mL} = Number\ of\ spores \frac{counted}{Counted\ area}\ X\ Chamber\ depth\ X\ Dilution$$

Ammonium nitrate

Ten grams of AP powder was taken in 250 mL conical flasks and 0.5 g of calcium carbonate was added in different concentrations of ammonium nitrate (0.5, 1, 2, 3 and 4 g) separately in different flasks. There were five treatments and each treatment was replicated thrice. Later the same methodology was followed as mentioned above.

pH

Ten grams of AP powder was taken in 250 mL conical flask then 40 mL distilled water was added in the flasks to make slurry. Sodium hydroxide (NaOH) or hydrochloric acid (HCl) of 1N concentration was added in the flasks to adjust the pH 6, 7, 8 & 10 and then same methodology was followed as mentioned above.

Temperature

As done for pH, 10 g of AP powder was taken in 250 mL conical flask and mixed properly with 40 mL distilled water using glass rod. All flasks were autoclaved at 121°C for 20 min and kept at different temperatures (15, 20, 25, 30 and 35°C). Further, the same methodology was followed as mentioned above.

Statistical analysis

The data on spore yield/production was analyzed by one way analysis of variance (ANOVA) using SPSS statistical software, version 16 and means were compared by Duncan multiple range test (DMRT).

Results & Discussion

Effect of addition of water in AP on spore production of EPF

Fig. 1A shows that 40 mL of water significantly improved (P <0.0001) spore production in *L. lecanii*, *B. bassiana* and *P. fumosoroseus* (50.53, 50.44 and 50.56 lakh spores/mL, respectively after 10 days of inoculation followed by 35 mL (46.30-47.53 lakh spores/mL) and 30 mL (45.01-45.33 lakh spores/mL). Spore production for 25 and 20 mL of water was at par with positive control (41.76-43.80 lakh spores/mL). The lowest spore production of all three EPF was observed in AP added with 15 mL of water (35.06-36.02 lakh spores/mL).

Effect of addition of ammonium nitrate

Addition of 4 g of ammonium nitrate in AP powder showed significantly (P <0.0001) high spore production in *L. lecanii*, *B. bassiana* and *P. fumosoroseus* (51.87-52.81 lakh spores/mL) after 10 days of inoculation and was followed by 3 g

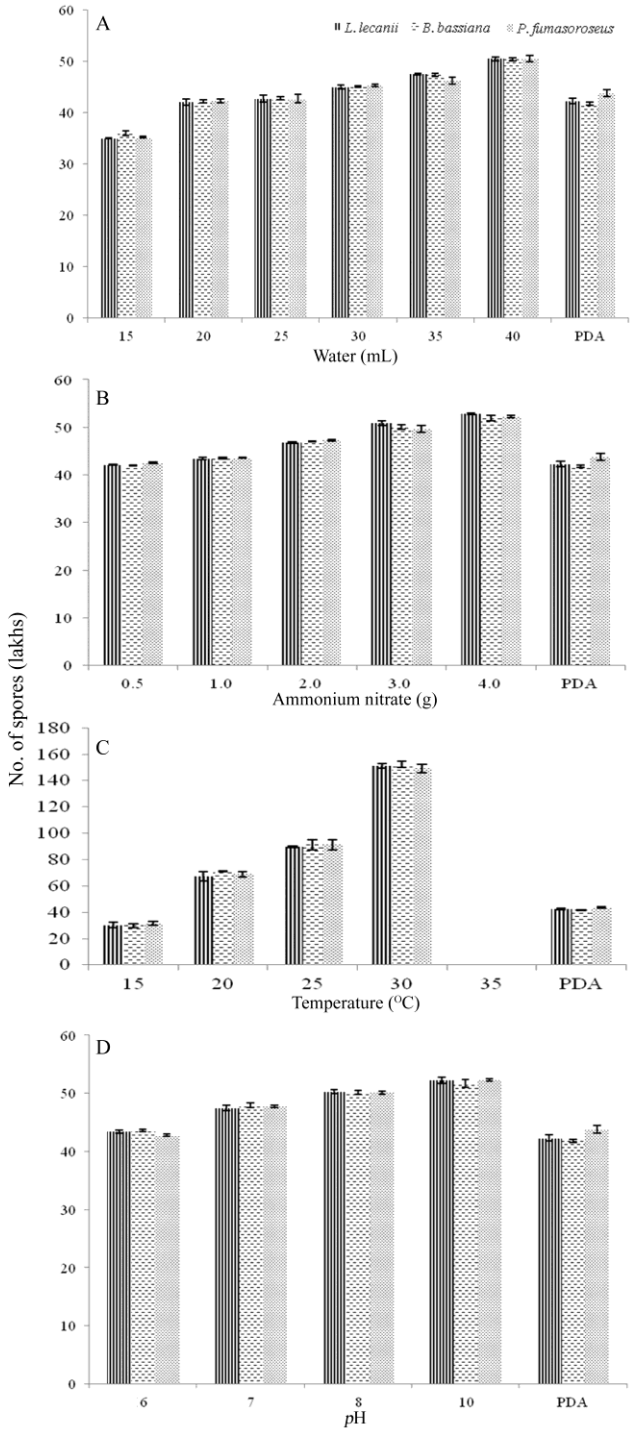

Fig. 1 — Effect of (A) water volume; (B) ammonium nitrate; (C) temperature; and (D) pH on spore production of entomopathogenic fungi in Apple Pomace.

(49.68-50.82 lakh spores/mL), 2 g (46.76-47.26 lakh spores/mL) and 1 g (43.51-43.58 lakh spores/mL) as shown in Fig. 1B. However, 0.5 g of ammonium nitrate resulted in comparatively less spore production (41.95-42.53 lakh spores/mL), at par with the positive control (41.76-43.80 lakh spores/mL).

Effect of temperature on spore production of EPF in AP

Fig. 1C which depicts the effect of different temperatures on spore production of *L. lecanii*, *B. bassiana* and *P. fumosoroseus* shows that, the spore production of EPF was significantly (P <0.0001) higher at 30°C (149.30-152.20 lakh spores/mL) after 10 days of inoculation and was followed by 25°C (89.79-91.24 lakh spores/mL) and 20°C (67.29-71.04 lakh spores/mL) as compared to PDA (149.30-152.20 lakh spores/mL).The lowest spore production was observed at 15°C (30.2 lakh spores/mL). No growth and spore production was seen at 35°C.

Effect of pH on spore production of EPF in AP

Effect of different pH on spore yield of *L. lecani*, *B. bassiana* and *P. fumosoroseus* multiplied on AP powder is presented in Fig. 1D. Spore production of EPF was significantly (P <0.0001) higher in alkaline pH of 8 and 10 (50.14-52.31 lakh spores/mL) and was followed by neutral pH 7 (47.47-47.95 lakh spores/mL) and acidic pH (42.78-43.58 lakh spores/mL). Spore production at acidic pH in *L. lecanii* and *P. fumasoroseus* was at par with positive control (41.76-43.8 lakh spores/mL).

The carbon and nitrogen plays major role for the growth and multiplication of EPF in solid state fermentation. Carbon source comes from the natural soluble/insoluble carbohydrates, while the nitrogen source is supplemented. Apple pomace contains sufficient fermentable carbohydrates that could be used as carbon source, but low level of nitrogen source available for EPF becomes the limiting factor for fungal growth. In the present study, addition of water, supplementation of ammonium nitrate in AP, adjusting the pH and effect of temperature directly influenced the growth and spore production in all the three entomopathogenic fungi, *L. lecanii*, *B. bassiana* and *P. fumosoroseus* after 10 days of inoculation. EPF prefer to grow more in alkaline pH as compared to neutral. AP has acidic pH (3 to 3.7) due to organic acid content, and therefore, it is necessary to add calcium carbonate to AP powder to neutralize AP medium. Due to acidic nature, the AP was adjusted with pH by adding HCl and NaOH and then reported better growth and spore production in alkaline pH as compared to acidic and neutral. In a similar study, yeast mold culture media adjusted with pH 5 to 9 and water activity (>0.99) showed better germination,

sporulation, growth and insecticidal activity of *Nomuraea rileyi*[5]. Growth rate and biomass of *B. bassiana*-G07 multiplied on yeast extract peptone glucose agar was higher in neutral to alkaline pH[6].

Moisture present in the media play significant role in the growth of fungi in solid media/substrate[7,8]. Higher moisture content in AP media increases the aerial mycelia. In the present study also, EPF showed significant growth when water was added to AP @ 3.5 to 4 mL of water/g. Similarly, supplementation of ammonium nitrate showed better growth of *L. lecanii*, *B. bassiana* and *P. fumosoroseus.* Present results were in conformity with the findings of Zheng & Shetty[8], who reported that addition of 0.05 g of calcium carbonate, 2 to 5 mL of water and 0.05 g of ammonium nitrate per gram of AP showed optimum growth in *Trichoderma* spp., *Penicillium* and *Rhizopus* species. Zheng & Shetty[9] also showed promising growth of *Trichoderma harzianum*, *Trichoderma pseudokoningii*, *Penicillium* isolate, and *Rhizopus* in cranberry pomace supplemented with calcium carbonate, water, and ammonium nitrate or fish protein hydrolysate/gram of pomace. In alignment with the observations of the present study, Reddy & Sahotra[10],also reported that addition of 40 mL water and 0.5 g of agar agar in 10 g AP medium at 5% showed promising growth of *L. lecanii* and was on par with AP 4 and 5%. The spore yield also was higher in AP 5% (16×10^6 spores/mL) as compared to other concentrations. The C:N (10:1) ration reported good growth and conidial yield (25×10^7 conidia/mL) of *B. bassiana* multiplied in Sabouraud dextrose yeast agar[11].

Temperature also influences growth and spore productionin EPF. In this study, 30°C showed more spore production, followed by 25°C. However, no growth was observed at 35°C. These results are in agreement with the findings of Cabonillas & Jones[12] who demonstrated optimal growth of *Isaria* sp. on Sabouraud maltose agar at 30°C and no growth at 35°C after 7 days of inoculation. Similarly, the growth of *B. bassiana* was also higher at 25-30°C (80-88%)[13] . In other study, growth rate and biomass of *B. bassiana*-G07 multiplied on yeast extract peptone glucose agar was higher at 25-27°C[6].

Though considerable literature is available on other agro-wastes/fruit wastes used for multiplication other fungi, only limited literature is there on application of AP waste for multiplication of EPF. Maximum spore production of *B. bassiana* was reported in vegetables and rice husk (10.76×10^8 spores/100 g)[14]. In another study, rice husk supported maximum spore production for *B. bassiana* (52×10^7spores/100 g) and *M. anisopliae* (59.5×10^7 spores/100 g)[15]. In the present study, the spore production of EPF was more in AP as compared to vegetable and rice husk. Molasses showed higher spore production of *B. bassiana* (6.9×10^{13} spore/mL)[16]. In a similar study, *P. lilacinus* recorded highest production of spores on biowaste of mango (3.3×10^7 spores/mL) followed by carrot, papaya and banana (3.2, 2.6 and 2.1×10^7 spores/mL, respectively). Similarly, *T. harzianum* produce maximum spore count on biowaste of carrot (3.14×107) as compared to mango chukandar banana and papaya (3.07, 2.97, 2.94 and 2.86×107 spores/mL, respectively)[17] as compared to tbhe present study. Vegetable waste reported maximum spore production of *M. anisopliae*, *Trichoderma longibrachiatum* and *B. bassiana* (8.8, 7.96 and 7.4×10^7 spores/g, respectively) after 15 days of incubation as compared to rice straw, sugarcane baggase, coconut coir and corn cob[18]. The production of β-glucosidase yield by *Penicillium verruculosum* was higher (45%) at 65°C and pH 4.5 using passion fruit peel as substrate[19]. In a similar study, Czapeck media supplemented with orange waste peel as carbon for *Aspergillus niger* showed higher production of enzymes. With partial optimization of culture, obtained maximum enzyme yield (117.1 ± 3.4 mu M/mL/min) at 30°C in an orange waste peel medium with pH 5.5 and 4% substrate concentration[20]. In another study, the growth of *Trichoderma atroviride* was faster as compared to *A. sojae*, and survival of *T. atroviride* in the tomato pomace was longer than *A. sojae*. Different carbon compounds were also shown to be produced by these two fungi on tomato and pepper pomaces[21]. The endophytic fungi grown on agro-industrial waste of pineapple peel (PP), sugarcane bagasse (SB) showed higher halo diameter of *Phlebia* sp. (15±0.16 mm) and of *Schizophyllum commune* (14.80±0.18 mm). Similarly, submerged cultures of PP or SB reported high a-amylase activity[22].

Conclusion

Apple pomace (AP) is a left-over waste after extraction of juice. Due to affordability and rich carbohydrate contents, AP was selected as a potential substrate for the growth and spore production of entomopathogenic fungi. The process was standardized by adding water, ammonium nitrate, modifying the pH and temperature @ 4 g/10 g AP),

temperature at 30°C and alkaline pH supported in maximum spore production of *L. lecanii* (50-52 lakh spores/mL), *B. bassiana* (50-152 lakh spores/mL) and *P. fumosoroseus* (50-149 lakh spores/mL). The results suggest that AP can be used as a solid substrate for mass production of EPF for commercial formulation.

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Conflict of interest

The authors declare no conflicts of interest.

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