Effect of High Inlet Temperature of Spray Dryer on Viability of Microencapsulated *Trichoderma asperellum* Conidia

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**Abstract.** In recent years, the use of spray drying for microencapsulating beneficial microbes has gained the interest of researchers, mainly due to dried powder formulation could prevent contamination and prolong self-life of the microbes. The major constraint of spray drying is conidia could lose viability during the drying process due to heat. In this present study, the effect of spray drying inlet temperature on viability of microencapsulated *Trichoderma asperellum* conidia was assessed. A blend biopolymer of gum Arabic and maltodextrin in ratio of 1:1 was used to microencapsulate the conidia at 150, 160 and 170°C inlet temperatures of spray drying process. Assessment of conidia viability was performed based on conidia percent survival of spray dried (PS<sub>sd</sub>) and survival increase (SI) unit values. Viability of the microencapsulated conidia was also evaluated their shelf life stored at two different temperatures which were at 28±2°C and 4±2°C for 40 weeks. The finding showed that viability of microencapsulated conidia was optimum obtained at inlet temperature of 170°C with 68.2% of PS<sub>sd</sub> and SI and 17.7 units of SI compared to 15.9% and 1.5 unit respectively obtained for 160°C and 0.2% and 0.7 unit for 150°C. The highest inlet temperature has showed the highest viability compared to lower temperatures. Conidia stored at low temperature of 4±2°C has survived longer up to 40 weeks that were confirmed via the viability test. High inlet temperature of 170°C was desirable to enhance survivability and viability of the conidia to be used as biocontrol agent up to 40 weeks at low temperature storage. These microencapsulated conidia could be further tested on their capability to inhibit the pathogen of pineapple disease.

**Keywords:** Spray Drying; Microencapsulation; *Trichoderma asperellum*; High Inlet Temperature

1. Introduction

Pineapple (*Ananas comosus* (L.) Merr) is a tropical fruit with sweeter taste, vibrant tropical flavour and enormous health benefits. It is the only important fruit crop in the family Bromeliaceae [1]. According to United Nations Conference on Trade and Development (UNCTAD), pineapple is the second in ranking of commercial tropical fruits for worldwide production after bananas. The high demands worldwide have risen the awareness to maximise the production of pineapple to meet the request. This includes minimising the pineapple crop lost due to the crop diseases caused by pathogenic fungi and bacteria. For a decade, *Trichoderma asperellum* has shown high potential as biological control agent...
(BCA) for many crop diseases such as for controlling cacao black pod disease in Cameroon [2], Fusarium ear rot of maize [3] and white rot disease of rubber [4]. Our in vitro study also revealed that *T. asperellum* is able to inhibit the growth of pathogenic fungus *Thielaviopsis paradoxa* the causal pathogen of black rot disease in pineapple fruits (data not publish). Therefore, the potential of this Trichoderma was further explored by developing a reliable formulation to enhance the capability this beneficial fungus as BCA for pineapple disease.

Microencapsulation achieved through a spray drying process could be the best method to maximise the potential of *T. asperellum* as BCA. Microencapsulation is a process of using tiny solid or liquid materials of active ingredient for coating with biopolymer materials to produce microcapsules through drying method [5]. The microcapsule could give various significant advantages, including an effective protection to the active ingredient against degradation and the ability to control the release rate of the active ingredient [6]. A spray drying is one of the drying technologies that commonly used nowadays since it has the lowest processing cost in a large-scale production as compared to a freeze drying method.

Various studies performing the spray drying have been reported using a variety number of inlet temperatures. Recent study performed by Braga et al. [7], reported that it was not possible to maintain the conidia viability when inlet temperature above 90°C even with the addition of wall material. This report is contradicting to our findings where we able to achieve high percent of conidia survival up to 70% with colony forming unit (CFU) x 10^7 of the encapsulated conidia. Although both studies are using the same fungus, *T. asperellum* as active ingredient, Braga used lower inlet temperatures of range between 60 to 120°C incorporated with higher dextrose-equivalent (DE) maltodextrin as biopolymer agent. In contrast, our study tested higher inlet temperatures of range between 150 to 170°C with lower DE maltodextrin as biopolymer agent. In addition, biopolymer used in this study is a blend of maltodextrin DE10 based a formulation with addition of gum Arabic that give a significant impact which will be further discussed in this paper. Other previous studies such as Jin and Custis [8] have used more wide range of inlet temperatures which were 40, 60, 80, 100, 120 and 140°C but they tested on *Trichoderma harzianum* incorporated with biopolymer agent of sucrose, molasses and glycerol. Meanwhile, Munoz-Celaya et al. [9] recommended the use of biopolymer coating agent mixtures of maltodextrin and gum Arabic for spray drying of *T. harzianum* under different sets of inlet temperatures which were 120, 135 and 150°C to give better result. Despite many studies has been reported using similar spray drying method with variety number of high inlet temperatures, using higher inlet temperature above 150°C for microencapsulating *T. asperellum* conidia specifically, has not been yet reported. Therefore, the objectives of this study were to produce microencapsulated *T. asperellum* conidia in maltodextrin-gum Arabic (1:1) biopolymer coating blend at three different high inlet temperatures of 150, 160 and 170°C, and to evaluate the viability of the encapsulated conidia produced with the different high inlet temperatures.

2. Materials and Methods

2.1 Strain, Culture Medium and Inoculum Development

*T. asperellum* was isolated from healthy leaves of pineapple variety MD2 collected from Kluang, Johor, Malaysia. The leaf samples were washed under running tap water for 20 minutes to remove any dirt from their surface. Both ends of the leaves were then discarded and remained in 7 cm in length of each leaf. Each leaf was cut into seven segments with 1 cm length in size per segment. All these leaf segments were surface sterilized using a slightly modified surface sterilization technique from Zaiton [10] by dipping in sterilized distilled water followed by 10% sodium hypochlorite and 70% ethanol for 1 minute each. The leaf segments were then rinsed twice with sterilized distilled water to remove all the possible chemical residues and allowed to dry on sterilized filter papers in a laminar flow chamber. The leaf segments were further recut into two small segments of 0.5 cm before transferred on potato dextrose agar (PDA) and then incubated for three days at 28 ± 2°C. All fungal mycelium grown out from the pineapple leaf tissues were isolated and purified into pure cultures before screening their potential as BCA against pineapple disease pathogen in *vitro* and in *vivo*. The fungal isolate with promising
antagonistic activity against the pathogen was selected and identified based on cultural and morphological characteristics and also confirmed by a molecular PCR amplification and phylogenetic analysis for strain identification. The genomic DNA result was referred to the National Center for Biotechnology Information (NCBI) BLAST against the GenBank sequence database and confirmed that the isolated fungus is *T. asperellum*. The culture of *T. asperellum* was then stored and maintained on half-strength PDA slants at 25°C and 4°C for further experiments.

2.2 Two-phase solid fermentation for conidial production

Prior to liquid fermentation phase, a pure culture of *T. asperellum* was grown on PDA at 30 ± 2°C for four days. This culture was necessarily needed for liquid fermentation. Yeast extract peptone dextrose (YEPD) broth was used in a 250 ml volume of conical flask to perform liquid fermentation. Three-days-old conidia of *T. asperellum* grown on PDA were taken out as a disc of 0.5 cm in diameter using a sterilized cork borer. Three discs were then inoculated into YEPD broth as an initial inoculum. The broth culture was wrapped with aluminium foil and left shaken in an incubator shaker at 150 rpm with temperature of 30 ± 2°C for four days. This fermented liquid culture was then used as a starter inoculum for a solid phase. The solid phase fermentation was performed by using 600 g rice chaff obtained from the Komplek Beras Bernas Seri Tiram Jaya, Tanjong Karang, Selangor, Malaysia. Each 600 g of rice chaff was formulated with 1 L distilled ionised (DI) water, 50 mL of molasses and 200 mgL⁻¹ (w/v) Chloramphenicol antibiotic. The rice chaff was soaked for 24 hours before inoculated with the liquid inoculum that was previously prepared. Each 100 g of soaked rice chaff was then placed into 1 litre beaker, and then injected evenly on the rice chaff surface with 15 mL of liquid inoculum to initiate solid fermentation process. The beaker was then wrapped with aluminium foil and incubated in an incubator at 30 ± 2°C for 25 days before the conidia were harvested.

2.3 Conidia Harvesting

After 25 days, conidia of *T. asperellum* were harvested from each beaker containing solid fermentation by soaking the solid with DI water containing 0.05% of Tween 80 for one hour. The soaked solid fermentation was then manually pressed and squeezed to dislodge the conidia from the rice chaff for five minutes. The available suspension in the beaker was filtered out by cotton gauze and then centrifuged at 2000 rpm for 15 minutes to collect the pellet of conidia. The collected conidia were stored in phosphate buffer solution (PBS) for further used in a spray drying experiment.

2.4 Biopolymer matrices

Laboratory grades of gum arabic (GA) (Nacalai) and maltodextrin (DE10) (Sigma) were used in a 1:1 ratio blend as the carrier for the spray-drying microencapsulation process.

2.5 Microencapsulation of conidia at different high temperatures

Aqueous solution of 20% (w/v) of GA-MD10 biopolymer and 1% (w/v) conidal suspension were both mixed in 500 mL of PBS and spray dried in a BUCHI Mini Spray Dryer B-290 model at the laboratory of International Islamic University Malaysia, Pahang, Malaysia. Each suspension (500 mL) was delivered to the dryer with an adjustment of the inlet air temperatures (Tᵢ) of 150°C, 160°C and 170°C, and the outlet air temperature (Tₒ) was fixed at 90°C ± 2°C.

2.6 Spray-drying of *T. asperellum* conidia formulation

The mixture of conidial suspension was sprayed in a spray dryer with an atomizer of 0.19-mm diameter operated with an air flow of 8.82 m³.min⁻¹, a pressure differential of 58.9 kPa, and the inlet (Tᵢ) and outlet (Tₒ) temperatures as stated previously were applied. The number of colonies forming units (CFU) of *T. asperellum* conidia before and after the spray drying process was determined by counting the CFU of conidial colonies on PDA plates. The percentage survival of the spray-dried (PSₚₛₜ) microencapsulated conidia was calculated using the following formula [9]:

\[
PS_{sd} = \frac{\text{CFU after spray drying}}{\text{CFU before spray drying}} \times 100
\]  

(1)
Meanwhile, the survival increase (SI) of microencapsulated conidia with respect to a non-encapsulated control treatment was obtained as follows:

\[
SI = \frac{PS_{sd} \text{ microencapsulated}}{PS_{sd} \text{ non-microencapsulated}}
\]  

(2)

2.7 Quantification of CFU count

Dry powder form of *T. asperellum* conidia was resuspended in PBS for 10 minutes and then diluted through a serial dilution method. Quantification of conidia based on CFU count was performed using Rose bengal media with chloramphenicol. The media contained 0.05 g L\(^{-1}\) of Rose bengal, 0.1 g L\(^{-1}\) of chloramphenicol, 10.0 g L\(^{-1}\) of dextrose, 5.0 g L\(^{-1}\) of Papai digest of soybean meal, 1.0 g L\(^{-1}\) of monopotassium phosphate, 0.5 g L\(^{-1}\) of magnesium sulphate, and 15.0 g L\(^{-1}\) of technical agar. Rose bengal and antibiotic Chloramphenicol were used in the media to inhibit unwanted bacterial growth. Rose bengal was added to the media to increase the selectivity and help to control the overgrowth of the selected fungus thereby assist in enumeration. Besides providing better isolation of slow-growing fungi, Rose bengal dye could be taken up by the fungal isolates, thereby aiding in their recognition. Smith and Dawson [11] found that the Rose bengal added to a near-neutral medium (pH of 6.8) has allowed for more colonies to develop than the acidified medium (pH of 4.2).

2.8 Effects of temperature on storage life of spray dried products of *T. asperellum*

This study is designed to evaluate the viability of spray dried inoculum of *T. asperellum* in maltodextrin DE10 based formulation with addition of gum Arabic against two different storage temperatures based on Table 1.

| Formulations        | Storage temperature (°C) | Label name |
|---------------------|--------------------------|------------|
| Maltodextrin 10DE + Gum Arabic | 27                        | GA         |
|                     | 4                        | GAC        |

The products of spray dried formulations were immediately divided into number of portions of 0.5 g each and stored inside arrays of airtight 2 mL centrifuge tubes. GA sample was stored inside an incubator, with temperature set at constant 27°C. GAC sample was stored inside refrigerator at 4°C. Both groups of tubes were contained inside an opaque box. For each experiment to be conducted, one tube will be consumed for every formulation and its contents were suspended in 1 L distilled water mixed with 0.002% (w/v) of tween 80, to aid in cells dispersion. 10 mL of suspensions were transferred inside universal bottle, shaken and let rest for 30 minutes to allow it soak and to further help loosen the clumps, and then shaken in vortex for another 30 seconds, again to ensure the suspension properly loosened into individual conidia. Using a hemocytometer (Neubauer-improved), the initial concentration was counted and serially adjusted into final concentration of 5 x 10\(^7\) cells/mL each. This concentration value was decided based on the preliminary result that suggested the concentration for optimal reading by plate counting at the end of experiment. For each formulation, 100 μL were plated with seven replications per each and the average number of colonies formed were recorded. Medium used was PDA with Chloramphenicol (200 mg/L) and Rose bengal (150 mg/L). PDA is used to promote quick growth of *T. asperellum*, Chloramphenicol (Bio Basic Canada Inc.) inhibits wide spectrum of undesired bacterial growth, while Rose bengal (Nacalai Tesque Inc., Japan) restricts the size of fast-growing *T. asperellum* colony, allowing for more opaque growth, in addition to providing contrast which both contributes to better visibility and clarity on plate counting. Inoculated plates were incubated for exactly 48 hours, at 28°C without parafilm sealing. Visibly formed colonies were then counted manually with aid of colony counter. Plates with less than 4 x 10\(^6\) cfu/mL average colony count were rounded as zero. This procedure was repeated every two weeks until all the tested formulations were exhausted of viable conidia. This experiment was carried out for 40 weeks.

2.9 Statistical analysis
An independent samples T-test was conducted to compare CFU count before and after microencapsulation. Three replicates of CFU counting were performed for each inlet temperatures to analyse $PS_{sd}$ and SI unit. Data were analysed using ANOVA with SPSS package.

3. Results and Discussion

3.1 Microencapsulation of conidia at different high temperatures

The effect of high inlet temperatures of spray drying on microencapsulated conidia of *T. asperellum* was evaluated based on the percentage survival of spray dried ($PS_{sd}$) by comparing the CFU count before and after the spray drying process. Theoretically, $PS_{sd}$ has a high percentage value when the difference of CFU between before and after the spray drying process is very small or no differences. In this study, the finding revealed that the value of $PS_{sd}$ was highest at 170ºC with up to 70% as shown in Table 2.

**Table 2.** Percentage survival of spray-dried ($PS_{sd}$) of encapsulated and non-encapsulated *T. asperellum* conidia at three different high inlet temperatures.

| Temperature (ºC) | Encapsulated (%) | Non-encapsulated (%) | Survival Increase (SI) |
|------------------|-------------------|-----------------------|------------------------|
| 170              | 68.2 ± 3.0        | 10.7 ± 2.6            | 17.7                   |
| 160              | 15.9 ± 4.7        | 3.9 ± 3.5             | 1.5                    |
| 150              | 0.2 ± 0.3         | 0.7 ± 0.4             | 0.7                    |

In contrast, the lowest $PS_{sd}$ was observed at 150ºC for the encapsulated *T. asperellum* conidia. Meanwhile, the non-encapsulated conidia $PS_{sd}$ have shown the same value pattern of encapsulated conidia with the highest $PS_{sd}$ value at 170ºC and lowest at 150ºC as shown in Figure 1.

![Figure 1. Effect of high inlet temperatures on the encapsulated and non-encapsulated *T. asperellum* conidia percent survival ($PS_{sd}$)](image)

The findings of this study suggested that conidia of *T. asperellum* were able to survive in the highest tested inlet temperature of 170ºC as compared to the lowest inlet temperature of 150ºC. This is possibly due to the shorter exposure time of thermal stress (during spray drying) to the spray-dried conidia, which was the outlet temperature. According to Fernandez-Sandoval *et al.* [12], thermal stress is a heat damage
occurred on the cellular membrane of conidia during a spray drying that can easily cause conidia to loss their viability. During the spray-drying process, the spray-dried conidia were exposed to the outlet temperature in the storage container while waiting for the whole process to end. However, the highest inlet temperature process gave the shortest time taken for the drying process to complete, that means the shortest exposure time of conidia to the heat of outlet temperature in the container. Hence, the 170ºC of inlet temperature gave higher PS_{sd} than PS_{sd} obtained at 150ºC inlet temperature high possibly due to less thermal stress.

The survivability of the conidia in high inlet temperatures also contributed by the addition of biopolymer coating of gum Arabic to base formulation of maltodextrin as well as the use of lower DE of maltodextrin. According to Takeiti et al. [13], morphologically, lower DE produce more voluminous maltodextrin particles and show smoother surfaces rather than higher DE that consist predominantly of globular shaped and partially encapsulated particles. In this case, the use of DE10 compared to DE20 mentioned earlier in introduction has resulted in smoother surfaces that showed complete encapsulation of the conidia as a microcapsule.

3.2 Survival Increase

Result of survival increase (SI) unit showed that the increment of conidia survival rate according to each inlet temperature. This indicated that a ratio value of percent survival (PS_{sd}) between the encapsulated and non-encapsulated conidia of T. asperellum at the same temperature. Higher percent survival of encapsulated conidia than non-encapsulated conidia would give high SI value. As shown in Figure 2, the SI unit at 150 to 160ºC are both in low ratio value. However, at 170ºC, the SI unit increased remarkably at 17.7 unit, resulting in the highest SI unit as compared to both 150 and 160ºC, 0.7 and 1.5 unit respectively. This result suggested that the highest temperature of 170ºC could provide a rapid formation of the microcapsules.

![Figure 2. Survival increase (SI) of encapsulated T. asperellum conidia with respect to a non-encapsulated control treatment at three different high inlet temperatures](image)

There was a huge difference of PS_{sd} value between the encapsulated and non-encapsulated conidia. Although both encapsulated and non-encapsulated conidia have shown the same pattern of increasing PS_{sd} value from 150ºC to 170ºC, however, PS_{sd} value of encapsulated conidia was clearly higher than
PS<sub>sd</sub> value of non-encapsulated conidia (Figure 1). This finding is very much similar to the previous research reports by Munoz-Celaya et al. [9]; Jin and Custis [8] mentioned that the microencapsulated conidia were higher survival percentage than non-encapsulated conidia. The higher survival percentage of encapsulated conidia is possibly due to the protective barrier of biopolymer matrices against the external factors such as thermal stress, dehydration and rehydration, and oxidative stress, while providing an intact environment for the conidia to prolong their shelf life. Thermal stress factor is less likely to be the heat exposure that could damage the conidia as mentioned previously. In this study, the biopolymer matrices of maltodextrin-gum Arabic blend were able to provide an excellent protection and physical barrier against the thermal stress produced during drying process for the encapsulated conidia. Furthermore, the dried powder form (dehydration) of <i>T. asperellum</i> conidia need to be rehydrated for field application. Both of these dehydration and rehydration can cause conidial cell damage and result in cell death [14]. According to Simonin et al. [15], dehydration and rehydration processes can cause changes in the permeability of the plasma membrane that is critically related to cell death. Therefore, the presence of biopolymer matrices resulted by the microencapsulation plays an important role in protecting the membranes of conidia from temperature during drying and rehydrating process. A previous study discovered that the trehalose content in <i>T. asperellum</i> conidia was related to the viability improvement during dehydration and rehydration processes [16]. Maltodextrin-Gum Arabic blend used in this study proved that the material can be applied to replace trehalose to protect the hydrophilic conidia of <i>T. asperellum</i> during the rapid spray drying through microencapsulation process. The gum maltodextrin-Gum Arabic blend solution that mixed with conidia in a suspension during spray drying had created an osmotic niche, and eventually formed a coating on the conidia.

A previous study by Perez-Alonso et al. [11] stated that those requiring higher activation energy (E<sub>a</sub>) during their drying process will provide a greater protection to the microorganism conidia from heat damage (thermal stress) and offer a greater resistance to oxygen diffusion (oxidative stress) through their drying matrices. Thus, high and suitable inlet temperature is needed to provide a complete encapsulated of conidia with maltodextrin-Gum Arabic blend through spray drying process. The inlet temperatures of 150ºC and 160ºC that resulted in low PS<sub>sd</sub> and SI unit might be due to inadequate heat energy received to achieve the activation energy level that could completely encapsulate the whole of conidia. The lack of heat energy caused inappropriate and uneven cover to encapsulate the whole of conidia, which could expose the conidia to thermal and oxidative stresses resulting in cells death.

### 3.3 Effects of temperature on storage life of spray dried products of <i>T. asperellum</i>

Our results show that spray dried product storing in refrigerated temperature of 4ºC may extend its lifespan by up to 90% compared to one stored in room temperature storage of 27ºC. At 27ºC, viability has been reduced to below 5 cfu x 10<sup>3</sup> mL<sup>-1</sup> by the 20<sup>th</sup> week. Storing in 4ºC has increased the shelf life of product more than 40 weeks (Table 3). There were no differences in growth rate and physical appearance of colony formed at any point along the lengths of study.

#### Table 3. Colony forming units (cfu) per ml of spray dried <i>T. asperellum</i> with formulations of maltodextrin and gum Arabic (GA) at 27ºC storage; and 4ºC storage respectively (GAC), at concentration of 5 x 10<sup>4</sup> cells/ml; Week 0 shows the counting of colonies right after spray drying process.

| Week | Cfu x 10<sup>6</sup> mL<sup>-1</sup> from 5 x 10<sup>7</sup> cells/mL suspensions |
|------|---------------------------------------------------------------|
|      | GA (27ºC)                  | GAC (4ºC)                     |
| 0    | 90.80 ± 3.00               | 90.80 ± 2.20                  |
| 2    | 78.30 ± 2.60               | 87.00 ± 4.70                  |
| 4    | 61.50 ± 2.80               | 78.30 ± 1.40                  |
| 6    | 38.00 ± 1.10               | 65.00 ± 2.10                  |
| 8    | 33.40 ± 4.90               | 59.70 ± 4.60                  |
|   | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 | 30 | 32 | 34 | 36 | 38 | 40 |
|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
|   | 23.80 ± 3.50 | 20.60 ± 3.70 | 18.00 ± 2.90 | 11.10 ± 3.80 | 8.20 ± 2.60 | 5.20 ± 1.70 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
|   | 55.50 ± 4.00 | 48.20 ± 2.60 | 44.00 ± 3.00 | 43.30 ± 2.60 | 39.40 ± 4.70 | 36.90 ± 2.80 | 33.00 ± 4.70 | 30.30 ± 4.90 | 24.30 ± 3.50 | 19.70 ± 2.90 | 19.00 ± 3.80 | 17.80 ± 4.60 | 14.20 ± 2.10 | 11.00 ± 3.00 | 7.40 ± 2.90 |

4. Conclusion

PS<sub>ad</sub> value and SI ratio at 170°C inlet temperature were highest than those in 150 and 160°C of inlet temperatures (p ≤ 0.05). The high temperature provides adequate energy to reach the activation energy level that encapsulates the conidia evenly and uniformly as a whole biopolymer microencapsulated conidia, in which it protects the conidia from the harsh external environment, thus prolong the shelf life of conidia.

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