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# EFFECT OF GROWTH-PROMOTING BACTERIA SUPPLEMENTED TO THE CASING LAYER ON THE YIELD AND MORPHOLOGICAL TRAITS OF AGARICUS BISPORUS

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Article history:		Abstract:			
Received: Accepted:	11 <sup>th</sup> May 2025 8 <sup>th</sup> June 2025	The study was conducted in November 2020 at the pioneering mushroom farm and laboratories of the College of Agriculture, University of Tikrit, to investigate the effects of bacterial isolates on the production of <i>Agaricus bisporus</i> mushrooms. Four bacterial isolates— <i>Bacillus pumilus, Pseudomonas fluorescens, Pseudomonas mosselii,</i> and <i>Pseudomonas putida</i> —were obtained from the laboratory and applied at three concentrations (10 <sup>6</sup> , 10 <sup>8</sup> , and 10 <sup>10</sup> cells ml <sup>-1</sup> ) two days after casing soil application.  The results revealed that <i>Pseudomonas putida</i> at a concentration of 10 <sup>10</sup> cells ml <sup>-1</sup> significantly outperformed all other treatments, recording the highest mushroom yield, biological efficiency, number of fruiting bodies, and stipe length, with increases of 21.24%, 20.99%, 25.62%, and 7.24%, respectively, compared to the control. Additionally, <i>Pseudomonas mosselii</i> at 10 <sup>8</sup> cells ml <sup>-1</sup> exhibited superiority in fruit body weight, cap thickness, and stipe diameter, with respective increases of 24.92%, 11.00%, and 21.05% over the control treatment.			

**Keywords:** Growth-promoting bacteria, Mushroom yield, *Agaricus bisporus*, Casing layer, Morphological traits

#### **INTRODUCTION**

In recent years, the consumption of the edible mushroom, A. bisporus, has increased worldwide due to its delicious taste, high nutritional value, and well-balanced nutritional components. This mushroom is known to contain essential amino acids, a substantial amount of vitamins, minerals, and dietary fiber, making them a valuable addition to the diet (Mattila et al., 2002; Ulziijargal et al., 2013). Moreover, A. bisporus has gained prominence as a key source of bioactive compounds with significant medical value, contributing to human health. Research has demonstrated their immune-boosting properties, anti-cancer effects, anti-inflammatory capabilities, cholesterol reduction, and prevention of liver diseases, heart ailments, hypertension, and liver diseases (Assemie and Abaya, 2022). Currently, China holds the global lead in A. bisporus production, accounting for 54% of the world's production, with a total annual yield of 2.37 million metric tons. The United States ranks second, contributing 9% to the global production, with an annual output of 409,000 metric tons, following closely are Poland, the Netherlands, India, France, and Spain (Royse et al., 2017).

The casing layer plays a pivotal role in the formation of fruit bodies in the A. bisporus mushroom, and without this layer, the fruit bodies may not develop. Several theories attempt to explain the role of the casing layer in triggering the transition from vegetative (mycelium) growth to reproductive or fruit stage, yet a precise explanation for this transformation remains elusive. Mushrooms require two essential components for fruit body formation: compost, which serves as a growth substrate, and the casing layer. The casing layer is prepared with suitable physical, chemical, and biological factors that induce the mushroom mycelium to form fruit bodies (McGee, 2018; Navarro et al., 2020). Both compost and the casing layer constitute a heterogeneous environment inhabited by a diverse range of microbial species, including bacteria, fungi, and viruses. While most studies have primarily focused on bacteria and fungi due to their significant roles in the production process, the bacterial community stands out as the more diverse and critical component, garnering greater attention in research compared to the fungal community (McGee, 2017). In a study conducted by Zarenejad et al. (2012), bacteria were isolated from 14 different casing soils, among which 23 bacterial isolates were identified as mushroom growth-promoting bacteria. Two of these isolates, belonging to Pseudomonas putida (Bt4 and Ps7), were found to be the most effective in promoting commercial-scale growth, resulting in the highest yield of fruit bodies. Murmu and Lal (2016) reported that the addition of P. putida to casing layers composed of various mixtures led to increased yield, vitality, and protein content compared to the control (without bacteria). It was found that bacteria belonging to the genera Pseudomonas and Bacillus have the ability to produce the hormones such as Indol

Acetic Aacid (IAA), which stimulates mycelial growth during the vegetative phase (Kang and Cho, 2014). In a study examining the effect of inoculating the casing layer with growth-promoting bacteria, researchers found that inoculating with P. putida and P. fluorescens led to a 26.62 and 9.07% increase in the A. bisporus yield, respectively (Rainey et al., 1990). Kim et al. (2008) demonstrated that using a strain of Pseudomonas bacteria to support Pleurotus eryngii mushrooms resulted in a 1.6-fold increase in growth, promoted the formation of primordia, reduced the number of days for fruit body appearance, and increased fruit body weight compared to the control. Lotfi et al. (2018) established a correlation between IAA production and fresh mushroom weight, as well as IAA production and the number of fruit bodies. Consequently, the study attributed the positive effects observed to the secretion of IAA and its growth-promoting influence. Moreover, growth-promoting bacteria present in the casing layer play an indirect role in enhancing fungal growth by inhibits potential pathogens in compost and casing layers (Braat et al., 2022).

This study aims to evaluate the efficacy of biological supplementation in the casing layer with bacteria isolated from different casing soils on the growth and yield of A. bisporus mushrooms.

#### **MATERIALS AND METHODS**

The experiment was conducted at the Mushroom Farm of the College of Agriculture, Tikrit University, on December 10, 2020, to investigate the impact of the growth-promoting bacteria into the casing layer on the yield and morphological characteristics of the fruit bodies produced by the edible mushroom A. bisporus.

# Stages of A. bisporus production;

# **Spawn Preparation**

Mushroom spawn was prepared according to the method of Hassan and Mahmoud (2003). Briefly; wheat grains were boiled in water, filtered, and supplemented with calcium sulfate at a rate of 2% and calcium carbonate at a rate of 8% of the dry weight of the grains. The mixture was distributed in glass bottles, then autoclaved at 121°C and a pressure of 1.5 kg/cm2 for one hour. The bottles were left to cool for 24 hours and subsequently inoculated with A. bisporus (strain A15) mycelial pieces. Finally incubated at 25±1°C until full mycelial growth was achieved.

#### **Preparation of Compost**

The composting process involved the mixing of 2000 kg of wheat straw with 1200 kg of poultry manure, 30 kg of calcium sulfate, and 10 kg of ammonium calcium nitrate. These components were moistened, and the fermentation process continued for two weeks with continuous turning. When the temperature reached approximately 60-70°C, the mixture was transferred to pasteurization chambers, and steam injection was initiated while ensuring adequate air circulation at a rate of 400 cubic meters per ton of compost per hour. On the second day, the temperature was raised to 60°C for 6 hours, and then it was gradually reduced from day 3 to day 6 until it reached 47°C. On the sixth day, once the ammonia odor disappeared, the temperature was lowered to 25°C, making the compost ready for use (Hassan and Mahmoud, 2003).

#### **Spawning Stage**

Two percent (2%) of the mushroom spawn was added to the fermented compost, thoroughly mixed, and packed into polyethylene bags measuring  $60 \times 40 \times 15$ cm (length x width x height). The bags were incubated at  $25 \pm 1$ °C for a period of three weeks.

### **Casing Stage**

Upon completion of mycelial growth, a casing layer consisting of peat moss, with a thickness of 4 cm, was applied. Relative humidity in the cultivation room was raised to 85%, while maintaining a temperature of approximately 25  $\pm$  1°C.

#### **Application of Growth-Promoting Bacteria**

The experiment involved the addition of four bacterial isolates: Bacillus pumilus, Pseudomonas fluorescens, Pseudomonas mosselii, and Pseudomonas putida. These isolates were taxonomically identified to the species level using the molecular method based on 16S rRNA gene sequencing (Hassan et al., 2022). Bacterial suspensions were prepared at three concentrations (610, 810, 1010 cells/mL) for each bacterial isolate. After two days of casing, the bacterial suspension was applied at a rate of 250 ml bag<sup>-1</sup>.

# **Cropping Stage**

During this stage, the relative humidity was reduced to 88%, and the carbon dioxide concentration was maintained at 1100-1300 ppm. The air temperature was maintained at approximately 18°C. Harvesting of the fruit bodies commenced five days after the appearance of primordia.

#### **Experimental Factors**

The experiment encompassed the addition of four bacterial isolates, namely B. pumilus, P. fluorescens, P. mosselii, and P. putida, at three concentrations, in addition to a control treatment, resulting in a total of 13 treatments:

- 1. B.p6: B. pumilus at 10<sup>6</sup> cells ml<sup>-1</sup>.
- 2. B.p8: B. pumilus at 10<sup>8</sup> cells ml<sup>-1</sup>.
- 3. B.p10: B. pumilus at 10<sup>10</sup> cells ml<sup>-1</sup>.
- 4. P.f6: P. fluorescens at 10<sup>6</sup> cells ml<sup>-1</sup>.
- 5. P.f8: P. fluorescens at 108 cells ml<sup>-1</sup>.
- 6. P.f10: P. fluorescens at 10<sup>10</sup> cells ml<sup>-1</sup>.
- 7. P.m6: P. mosselii at 10<sup>6</sup> cells ml<sup>-1</sup>.
- 8. P.m8: P. mosselii at 10<sup>8</sup> cells ml<sup>-1</sup>.
- 9. P.m10: P. mosselii at 10<sup>10</sup> cells ml<sup>-1</sup>.

- 10. P.p6: P. putida at 10<sup>6</sup> cells ml<sup>-1</sup>.
- 11. P.p8: P. putida at 108 cells ml-1.
- 12. P.p10: P. putida at 10<sup>10</sup> cells ml<sup>-1</sup>.
- 13. Control (without bacteria).

#### **Experimental Parameters**

# Number of fruit bodies (Fruit bodies bag<sup>-1</sup> (experimental unit<sup>-1</sup>)

Calculated by counting the total number of fruits produced in each experimental unit from three flushes.

#### Total yield (q baq<sup>-1</sup>)

Total yield calculated by summing the yield obtained from each experimental unit (bag) of three flushes and expressed as g bag<sup>-1</sup> (contain 15 kg of compost)

#### Average fruit body weight (g fruit body-1)

Calculated using the following equation:

Average fruit body weight = Total weight of fruit bodies produced from one bag / Number of fruit bodies produced from one bag

#### **Biological Efficiency (B.E) (%)**

This metric evaluates the strain's efficiency in production relative to the amount of the growing medium (compost). It is calculated using the formula:

B.E (%) = [Total weight of fruit bodies (g)]  $\times$  100 / [Dry weight of the compost at spawnning Application (g)].

#### **Morphological Traits (cm)**

Morphological measurements included the length and diameter of the stipe, , thickness and diameter of the cap, which were measured using vernier calipers.

#### **Statistical Analysis**

The experiment was designed according to a completely randomized design (CRD). The data was statistically analyzed using the SAS (Statistical Analysis System) software and the means were compared according to the Duncan's Multiple Range Test at a significance level of 0.05 (Rawi and Khalafallah, 2000).

#### **RESULTS AND DISCUSSION**

# The impact of the growth-promoting bacteria on the productive traits of the mushroom A. bisporus

The results presented in Table (1) indicate variability among the studied treatments with regard to the effects of bacterial isolates and their different concentrations on the production traits of A. bisporus. The treatments Pp10, Pp8, Bp8, and Pm10 recorded the highest fruit body yield, reaching 6616.08, 6174.07, 6109.13, and 6082.98 g bag<sup>-1</sup>, respectively. These treatments showed statistically significant differences compared to the remaining treatments and the control treatment, which recorded a total yield of 5210.60 g bag<sup>-1</sup>. Conversely, the Pm6 treatment exhibited the lowest fruit body yield at 5157.11 g bag<sup>-1</sup>. From the same table, it is evident that the Pp10 recorded the highest number of fruit bodies, reaching 242 fruit bodies bag-1, showing statistically significant differences from all other treatments with an increase of 15.70% compared to the Pp8 treatment, which followed with 204 fruit bodies bag-1, while the lowest number of fruit bodies was 122 fruit bodies bag<sup>-1</sup>in Pm8 treatment. Regarding the average fruit body weight, Pm8 showed superiority, with a weight of 38.57 g fruit body<sup>-1</sup>, followed by the Pp6 treatment at 35.64 g fruit body<sup>-1</sup>, while the Pp10 treatment ranked last, with a weight of 27.40 g fruit body<sup>-1</sup>. In terms of biological efficiency (B.E), the table reveals that the treatments Pp10, Pp8, Bp8, Pm10, and Bp10 recorded the highest biological efficiencies at 81%, 76%, 75%, and 71% with percentage increases of 20.99%, 15.79%, 15.79%, 14.67%, and 9.86% over the control, respectively, on the other hand, Pf8 treatment recorded the lowest biological efficiency at 62%.

The results of this experiment are in line with the findings of numerous researchers. For example, in a study conducted by Zarenejad et al. (2012), about 23 growth-promoting bacterial isolates were identified from 14 different casing soils, two of which belonged to Pseudomonas putida (Bt4 and Ps7) and were found to be the most beneficial for commercial mushroom growth, yielding the highest fruit body production. Additionally, Kim et al. (2008) demonstrated that the use of the Pseudomonas strain P7014 to support P. eryngii led to a 1.6-fold increase in growth, stimulated the growth of primordia, reduced the time to appearance, and increased the weight of fruit bodies compared to the control .

In another study, researchers found that inoculating the casing layer of the A. bisporus mushroom with P. putida and P. fluorescens resulted in an increase in yield by 26.62% and 9.07% respectively, although the latter result was not statistically significant (Rainey et al., 1990). Cho et al. (2003) also reported that inoculating the mushroom casing layer with P. fluorescence stimulated the formation of primordia and hastened their maturation.

The preceding table reveals a significant incremental enhancement in fruit body yield, biological efficiency, and fruit body count with the augmentation of the bacterial isolates P. putida, and these characteristics reached their highest levels in the Pp8 and Pp10. This can be attributed to the symbiotic interaction of these bacteria with fungal hyphae in the casing layer and compost, which have the benefit of reducing volatile substances such as ethylene produced by fungal hyphae during growth. These substances inhibit primordia formation. Consequently, these bacteria are considered fruit body promoters (Chen et al., 2013). Noble et al. (2009) affirmed that P. putida bacteria adhere to fungal hyphae and consume the volatile organic compound,1-octen-3-ol (a promoter of vegetative growth and inhibitor of fruit), thereby promoting fruit body formation.

The increase of A. bisporus yield by P. putida (Pp8 and Pp10), as well as Bp8 and Pm10 attributed to B. pumilus and P. mosselii, can be traced back to the direct nourishment of A. bisporus on these bacteria and their constituents. This contributes to fungal hyphae growth, fruit body formation, and increased mushroom yield, a phenomenon corroborated by Vos et al. (2017), who observed a reduction of bacterial biomass by 75% during the production stage, compensating for an increase in fruit bodies. This suggests direct nourishment of the fungus on bacteria and their constituents in the later stages of fruit body production.

Many types of bacteria release growth-promoting substances that positively influence production characteristics. Bacteria belonging to the Pseudomonas and Bacillus genera have been found to secrete the hormone IAA (indole-3-acetic acid), which stimulates fungal hyphal growth during the vegetative stage (Kang and Cho, 2014). This has a positive impact on increasing the number of fruit bodies, fresh and dry weight, protein content, and cap diameter (Mohammad and Sabaa, 2013). Lotfi et al. (2018) affirmed a correlation between IAA production and fresh fungal weight on one hand, and IAA production and the number of fruit bodies on the other, attributing the positive impact observed in the studied characteristics to the secretion of IAA and its stimulating effect on mushroom growth. Moreover, Young et al. (2013) demonstrated the ability of these bacterial species to fix nitrogen and solubilize phosphate, further promoting growth and reducing the number of days required for primordia formation. Additionally, growth-promoting bacteria present in the casing layer have an indirect effect on fungal tissues and growth enhancement. They inhibit microorganisms causing mushroom diseases, such as bacteria or fungi commonly found in the agricultural environment and casing layer (Braat et al., 2022). Some of them also have the ability to combat diseases associated with fungal growth. Several bacterial species distributed in the casing layer are known to control bacterial blotch disease, which is prevalent in production facilities, and reduce the infection rate, which can sometimes reach 100%, as with P. putida, P. fluorescens, and P. reactans (Ghasemi et al., 2020).

Table (1) The effect of growth-promoting bacteria on the productive traits of the mushroom A. bisporus

	Mushroom yield					
Treatments	Total yield (g bag <sup>-1</sup> )	Number of fruit bodies (fruit bodies bag <sup>-1</sup> )	Fruit body weight (g fruit body <sup>-1</sup> )	Biological efficiency (%)		
Вр6	5234.62	168	31.37	65		
	d d-f c-e		c-e	d		
Bp8	6109.13	201	30.28	76		
	a-c	bc	de	ab		
Bp10	5785.20	174	33.33	71		
Бріо	b-d	c-f	bc	a-d		
Pf6	5370.46	177	30.32	67		
	b-d	b-f	de	cd		
Pf8	5002.00	157	31.88	62		
FIO	d	e-g	cd	d		
Pf10	5662.16	193	29.29	70		
	b-d	b-d	d-f	b-d		
Pm6	5157.11	164	31.50	63		
FIIIO	d	e-g	c-e	d		
Pm8	5300.91	137	38.57	65		
	cd	g	a	cd		
Pm10	6082.98	182	33.57	75		
	a-c	b-e b		a-c		
Pp6	5298.79	149	35.64	65		
. 50	cd	fg	b	cd		
Pp8	6174.07	204	30.27	76		
ι ρο	ab	b	de	ab		

Pp10	6616.08	242	27.40	81
1 10	a	a	f	a
Control	5210.60	180	28.96	64
Control	d	b-e	ef	d

<sup>\*</sup>Similar letters indicate that there are no significant differences according to Duncan's multiple test at the probability level of 0.05.

# The impact of the growth-promoting bacteria on the morphological characteristics of *A. bisporus* fruit bodies

Results from Table 2 indicate that the highest cap diameter was achieved by Pm6 and Bp10, measuring 6.45 and 6.41 cm, respectively, followed by Pm10 with a cap diameter of 6.35 cm. In contrast, Pp10 recorded the lowest cap diameter at 5.88 cm. From the same table, Pm8 recorded the highest cap thickness at 2.91 cm, followed by Pm6 with a cap thickness of 2.84 cm, while the lowest cap thickness was 2.21 cm in Pf10 treatment. Regarding stem diameter, the results show that Pm8 recorded the highest stem diameter at 2.66 cm, followed by 2.47 cm by Bp10 compared to lowest stem diameter at 1.79 cm by Pp10.

Pp10 and Pf8 had the longest stipes, measuring 2.90 and 2.74 cm, respectively, followed by the control with a stipe length of 2.69 cm. However, The shortest stipe lengthwas 2.11 cm in treatment of Pm6. It is noteworthy from the table 1 results that treatments with fewer fruit bodies exhibited better characteristics in terms of cap diameter, cap thickness, stipes diameter, and stipes length. The correlation in table 3 indicates a positive relationship between fruit body weight and each of stipes diameter, cap diameter, and cap thickness, with correlations of 0.294, 0.342, and 0.419, respectively. This may be attributed to the lower number of fruit bodies and reduced crowding within the unit area, leading to increased nutrient availability and improved ventilation in the surrounding environment. Consequently, the fruit bodies in these treatments experienced optimal growth. Conversely, Pp10, which involved the addition of *P. putida* at a concentration of 10<sup>10</sup> cells/mL, directly or indirectly stimulated the formation of primordia (as discussed in the production characteristics), resulting in increased crowding within the unit area. This competition for nutrients, along with reduced oxygen and increased carbon dioxide levels, led to smaller caps and longer stipes for the fruit bodies, as well as a reduced fruit body weight (27.40 g). This observation aligns with the negative correlations identified in table 3 between cap diameter and stipes length (-0.346) and between fruit body weight and the number of fruit bodies (-0.740).

These findings are in agreement with previous studies by Kivaisi (2007) and Nayak et al. (2015), which suggested that an increase in carbon dioxide concentration leads to longer stipes and occasionally irregularly shaped fruit bodies. Additionally, the measurements of cap diameter and thickness, as well as stipes diameter, align with the findings of Eren (2022). However, they differ from Eren's results regarding stipes length, stipes diameter, and cap thickness.

Table (2) The effect of growth-promoting bacteria on the morphological characteristics of the mushroom A. bisporus

	Morphological characteristics					
Treatments	Cap diameter (cm)	Cap thickness (cm)	Stipe diameter (cm)	Stipe length (cm)		
Bp6	6.24	2.62	2.30	2.64		
	a-c	a-c	a-d	a-c		
Bp8	6.32	2.62	2.38	2.45		
	ab	a-c	a-d	a-c		
Bp10	6.41	2.76	2.47	2.44		
	a	ab	ab	a-c		
Pf6	6.20	2.44 1.79		2.68		
	a-c	bc	d	a-c		
Pf8	6.30	2.55	1.89	2.74		
	a-c	a-c	cd	ab		
Pf10	6.30	2.21	1.97	2.56		
FIIO	a-c	С	b-d	a-c		
Pm6	6.45	2.84	2.47	2.11		

	a	ab	a-c	С
Pm8	6.34	2.91	2.66	2.29
	ab	a	a	bc
Pm10	6.35	2.67	2.23	2.20
1 11110	ab	ab	a-d	bc
Pp6	6.12	2.56	1.92	2.28
1 60	ab	a-c	bcd	bc
D0	6.05	2.49	1.81	2.62
Pp8	a-c	bc	cd	a-c
Pp10	5.88	2.43	1.79	2.90
	С	bc	d	a
Control	5.97	2.59	2.10	2.69
Control	bc	a-c	a-d	a-c

<sup>\*</sup>Similar letters indicate that there are no significant differences according to Duncan's multiple test at the probability level of 0.05.

Table (3) The simple correlation between yield characteristics, its components, and phenotypic characteristics of the mushroom A. bisporus

eristics of the mushroom A. disporus							
	Number of	Total	Fruit body	Stipe	Stipe	Cap	Cap
	fruit bodies	yield	weight	length	diameter	diameter	thickness
Number of fruit bodies							
Total yield	0.828**						
Fruit body weight	-0.740**	-0.249					
Stipe length	0.399*	0.267	-0.358*				
Stipe diameter	-0.282	-0.159	0.294	-0.352*			
Cap diameter	-0.345*	-0.178	0.342*	-0.346*	0.318*		
Cap thickness	-0.340*	-0.158	0.419**	-0.332*	0.373*	0.330*	
Biological efficiency	0.830**	0.997**	-0.257	0.254	-0.180	-0.191	-0.171

#### CONCLUSION

In conclusion, among the four bacterial isolates tested, *Pseudomonas putida* at a concentration of  $10^{10}$  cells ml $^{-1}$  demonstrated superior performance in terms of yield, biological efficacy, number of fruit bodies, and stipe length for the mushroom A.bisporus, exhibiting significant percentage increases compared to the control treatment. Additionally, Pseudomonas mosselii at a concentration of  $10^8$  cells ml $^{-1}$  outperformed other treatments in fruit body weight, cap thickness, and stipe diameter, with substantial percentage enhancements over the control treatment. These findings highlight the potential of these bacterial isolates for enhancing mushroom cultivation outcomes.

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