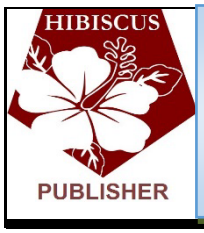
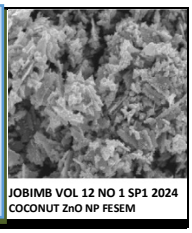
JOBIMB, 2024, Vol 12, No 1 SP1, 57-60
https://doi.org/10.54987/jobimb.v12iSP1.943



JOURNAL OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY
Website: http://journal.hibiscuspublisher.com/index.php/JOBIMB/index



Microbial Characterization of *Belacan* (Fermented Shrimp Paste) and Its Potential as a Plant-Growth Promoting Agent of Oyster Mushroom (*Pleurotus ostreatus*)

Khudair J.D. Abedelazeez[1], Nurul Solehah Mohd Zaini[1], Nur Aisyah Syahirah Ahmad Fauzi[1], Nurazlin Zainuddin[1], Tiun Xin Ci[2], Hosynaa Elang Kesvaran[2], Jamilah Syafawati Yaacob[2] and Muhamad Hafiz Abd Rahim[1]*

[1]Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[2]Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia.

*Corresponding author:
Muhamad Hafiz Abd Rahim
Department of Food Science,
Faculty of Food Science and Technology,
Universiti Putra Malaysia,
43400 UPM Serdang,
Selangor,
Malaysia.
Email: muhdhafiz@upm.edu.my

HISTORY

Received: 7th April 2024
Received in revised form: 5th July 2024
Accepted: 30th July 2024

KEYWORDS

Belacan
Plant-growth-promoting bacteria
Mushroom
Biofertilizer
Organic farming

ABSTRACT

Belacan, or fermented shrimp paste, is produced through the fermentation of small shrimps and salts under specific conditions. As a result of spontaneous fermentation, *belacan* harbors a diverse microbial community comprising bacteria, fungi, and yeast. These microorganisms possess the potential to influence not only human health but also plant health. Hence, this project seeks to elucidate the bacterial characteristics of *belacan* in promoting the growth and development of oyster mushrooms (*Pleurotus ostreatus*). Through the identification and isolation of two prominent bacterial strains, *Lysinibacillus fusiformis* and *Bacillus velezensis*, it has been revealed that these bacteria can benefit plant growth. Further investigation has demonstrated that these bacteria serve as plant growth-promoting bacteria (PGPB), exhibiting positive effects on phosphate solubilization, indole-3-acetic acid (IAA) levels, antifungal activity, and the physical characteristics of oyster mushrooms.

INTRODUCTION

Belacan, also known as shrimp paste, is a traditional Southeast Asian condiment that holds a significant place in the culinary cultures of countries such as Malaysia, Indonesia, Thailand, Vietnam, and the Philippines. *Belacan* is typically made by fermenting small shrimps or krill. The process involves salting the shrimps and then allowing them to undergo natural fermentation [1]. The fermentation process yields a diverse array of indigenous microorganisms (IMO), enriching the product with unique organoleptic properties and potential applications beyond culinary uses, such as fostering plant growth. Curiously, these fermented foods were touted to also contributed to the enhancement of plant growth by promoting plant growth, enhancing nutrient uptake, and increasing tolerance to environmental stresses [2]. These properties are mainly contributed by the presence of indigenous microorganisms (IMO). IMO is known to possess bacteriocins (inhibit plant pathogens), increase the solubilization of plant macronutrients such as Nitrogen (N), Phosphorus (P), and Potassium (K), and produce phytohormones, such as indole acetic acid (IAA) or auxin [2]. Currently, the local practice often utilizes these fermented food substrates on the important agricultural crops, such as chilli (*Capsicum* spp.).

To improve highly sustainable crops like oyster mushroom, fermented fish products like *belacan* that contain beneficial compounds like amino acids, peptides, and vitamins may be used to promote mushroom growth [2]. These nutrients provide essential elements for mycelium development, while growth factors stimulate growth. This project seeks to investigate the bacterial traits of *belacan* that promote the growth and development of oyster mushrooms (*Pleurotus ostreatus*).

MATERIALS AND METHOD

Preparation of belacan-molasses mixture

For the preparation of *belacan*-molasses mixture, *belacan* was mixed with pure sugarcane molasses (Bio Terra Solutions Sdn Bhd, Malaysia) and water with a ratio of 1:1:1 in the universal bottle. The mixture was kept for fermentation for 7 days to enrich beneficial microbial activity.

Phosphate solubilisation test

The bacteria were centrifuged at 10,000 rpm for 5 min and 60 µL of supernatant bacteria was pipette into the well of Pikovskaya agar. The procedure was conducted in sterile conditions in which laminar flow. The halo zone (inhibition zone) was measured in a two-day interval for a total of 8 days of the incubation of plates at 28 °C.

Antifungal test (Well diffusion)

A 10 mL of nutrient agar was poured into each plate followed by potato dextrose agar. The agar plate surface is inoculated by spreading 100 µL of the fungi over the entire agar surface. Then, a well with a diameter of 6 to 8 mm is done aseptically with a sterile cork borer, and 60 µL of the bacteria with the desired concentration is introduced into the well. Finally, the agar plates are incubated under 30 °C. The bacteria diffuse in the agar medium and inhibit the growth of fungi.

Indole-3-acetic acid (IAA) Quantification Methods Using Salkowski Reagent

Salkowski reagent was prepared by mixing 600 µL of 0.5 M iron (III) chloride with 30 mL of 35% perchloric acid. Bacterial stock cultures (1 mL) were centrifuged at 16300 ×g for 5 min, and the supernatants were transferred to new tubes. Then, 100 µL of Salkowski reagent was added to a 96-well plate, followed by 100 µL of supernatant. After thorough mixing, the formation of a pink color indicated IAA production, and absorbance was measured at 530 nm using a Gen 5 BioTek Epoch Microplate Spectrophotometer. A standard curve was generated using different concentrations of IAA (10-100 µg/ml) in distilled water, allowing estimation of IAA concentrations produced by each bacterial culture [3].

Treatment, Growing, and Harvesting of mushrooms (*Pleurotus ostreatus*)

Oyster mushrooms (*Pleurotus ostreatus*) were cultivated using wooden husks at the Mushroom House of Bukit Ekspo, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The facility is equipped with ventilation and humidification systems. Mushroom seeds were inoculated into the blocks, which were then placed in the incubation room for 2.5 to 3 months. Different treatments were applied during the incubation period. To facilitate mushroom production, the covers were opened, ensuring the sponge was removed from the block's mouth. Blocks were watered twice daily, with care taken to prevent water from entering the block's mouth. Harvesting occurred 4-5 days after opening the cover, with entire clusters pulled out for harvesting.

Statistical analysis

Data obtained was recorded by using Google Sheets and analyzed using the Minitab Software Version 2.1. One-way analysis of variance (ANOVA) with Single Factor and Tukey's test was used to compare the means when a significant variation was established by ANOVA at the significance level 0.05 ($P<0.05$).

RESULT AND DISCUSSION

Concentration of bacteria (CFU/mL) at different optical densities (OD)

The correlation between OD and concentration of bacteria as a preliminary step was presented in **Table 1**. The purpose of this analysis was to standardize the initial working concentrations for diverse treatments as bacterial strains in subsequent analysis; phosphate solubilization analysis, determination of IAA levels, antifungal activity, well diffusion analysis, and application to oyster mushroom cultivation. *Lysinibacillus fusiformis* and *Bacillus velezensis* show an increase in concentration as the optical density (OD) increases, indicating growth. The higher the OD, the higher the concentration of the bacteria obtained.

Table 1. The concentration of bacteria (CFU/mL) at different optical densities (OD).

Concentration of bacteria (OD = Optical Density)	Bacterial concentration (CFU/mL)	
	Lysinibacillus fusiformis	*Bacillus velezensis*
OD_{600nm}= 0.1	1.14×10^6	6.90×10^6
OD_{600nm}= 0.2	3.04×10^6	4.20×10^6
OD_{600nm}= 0.3	1.29×10^6	3.00×10^6
OD_{600nm}= 0.4	1.49×10^6	1.70×10^7
OD_{600nm}= 0.5	3.47×10^6	3.10×10^7
OD_{600nm}= 1.0	6.40×10^7	1.37×10^7

Phosphate solubilization of different treatments

Based on **Fig. 1**, *Lysinibacillus fusiformis* and *Bacillus velezensis* have the capability to solubilize phosphate, as indicated by the presence of an inhibition zone on the Pikovskaya agar (**Fig. 2**). The inhibition zone of *Lysinibacillus fusiformis* and *Bacillus velezensis* showed an increasing trend after 8 days of observation, but was not significant in terms of sizes, signifying that the solubilization of phosphate is not affected by time. Furthermore, the halo zones of both bacteria were insignificant to each other, demonstrating their comparable phosphate-solubilization abilities [4]. Nevertheless - *Belacan*, positive control (EM solution), and negative control (water) produced no halo zones compared to *Lysinibacillus fusiformis* and *Bacillus velezensis*, which indicated that they do not solubilize the phosphate.

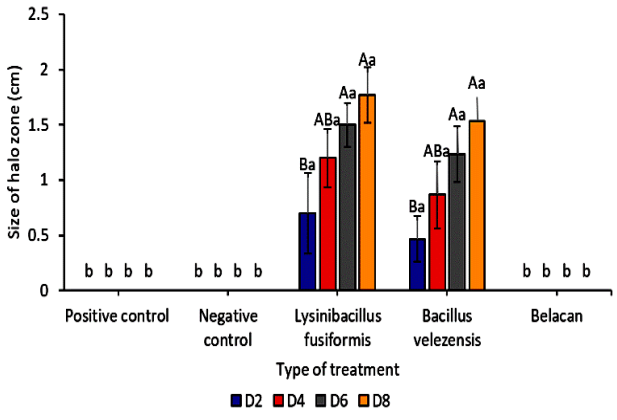


Fig. 1. Size of the inhibition (halo) zone of different types of bacteria at interval day. Data were expressed in mean ± standard deviation. Different uppercase letter indicates significant differences within the size of the halo zone of the same treatment ($p<0.05$). Different lowercase letter indicates significant differences within the size of the halo zone of different treatment ($p<0.05$).

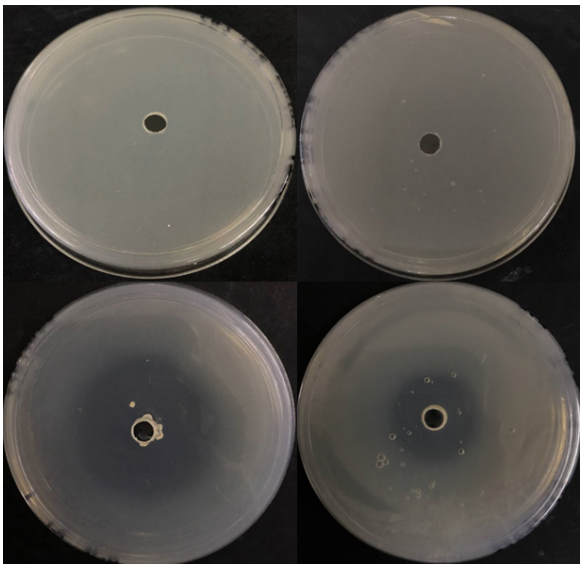


Fig. 2. Halo zone of treatments on Pikovskaya agar. From upper left: positive control (EM solution), negative control (water), *Lysinibacillus fusiformis*, and *Bacillus velezensis*. The figure is not drawn/shown to scale.

Inhibition zone of Trichoderma on different treatments

In commercial mushroom cultivation, the growth of the green fungus *Trichoderma* often led to mushroom growth retardation. In this investigation, *Lysinibacillus fusiformis*, *Bacillus velezensis,* and Amphotericin B showed statistically significant inhibition zones of *Trichoderma* on potato dextrose agar ($p<0.05$) in **Fig. 3**. Interestingly, both bacterial strains demonstrated similar inhibition capacity with a known fungal inhibitor Amphotericin B, and a much greater inhibition zone than positive control (EM). *Bacillus velezensis* had the highest inhibition zone which is in agreement with results reported by Oana Alina et al. [5]. The study showed that *Bacillus velezensis* strains significantly suppressed the mycelial growth of all pathogenic fungi (ranging from 40.0% to 87.4%) compared to the controls. *Belacan* only, was insignificant with positive and negative controls. The value indicated that *belacan* had inhibition properties but was less effective compared to the singly bacterial treatments. The mixture of various microbial species (e.g. in *belacan*) may not be optimal for antagonistic activities of *Trichoderma* [6].

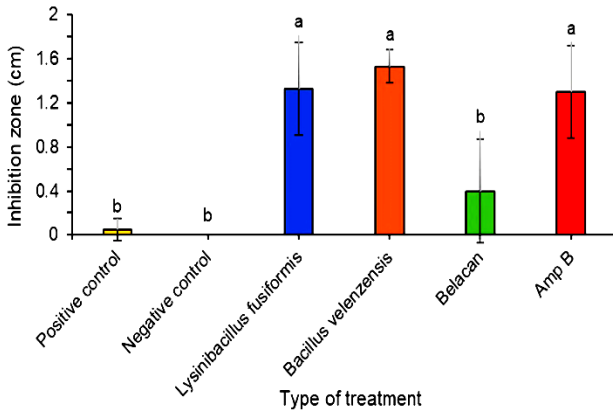


Fig. 3. Inhibition zone of *Trichoderma* on different treatments. Data were expressed in mean ± standard deviation. Different lowercase letter indicates significant difference ($p<0.05$).

Indole-3-acetic acid (IAA) levels in different treatments

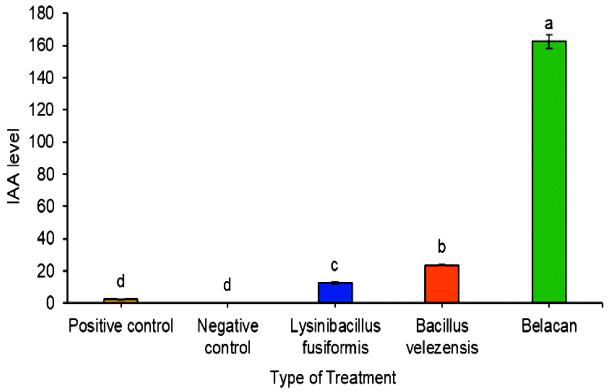


Fig. 4. Indole-3-acetic acid (IAA) levels in different treatments. Data were expressed in mean ± standard deviation. Different lowercase letter indicates significant difference ($p<0.05$).

IAA level in different treatments is illustrated in **Fig. 4**. Both *Lysinibacillus fusiformis* and *Bacillus velezensis* contained a significant level of IAA, as indicated by the statistical significance relative to the controls. However, *Bacillus velezensis* has a significantly higher level of IAA compared to *Lysinibacillus fusiformis*. The positive control had a low level, while negative control had no detectable IAA. Interestingly, *belacan* had a significantly higher IAA, which surpassed other treatments and controls. This is likely because *belacan* may contain substances or compounds that naturally have a high IAA content or promote IAA production, as demonstrated in a previous study by Shi et al. (2017)[7]. During the fermentation process, certain microorganisms produce biostimulants to produce plant hormones (auxins, cytokinins, ethylene, gibberellins, and abscisic acid) which contribute to plant growth [7].

Wet weight of mushrooms with different treatments

Based on **Fig. 5**, the wet weight of mushrooms treated with *belacan*, *Lysinibacillus fusiformis* and *Bacillus velezensis* have significantly higher wet weight compared to positive and negative control mushrooms. Previous research by Verma et al. (2015) showed that *Lysinibacillus fusiformis* can convert fixed organic forms of phosphorus into easily soluble P forms that are simple for plants to absorb, and the efficient and maximum absorption contributes to higher yield of oyster mushrooms [8].

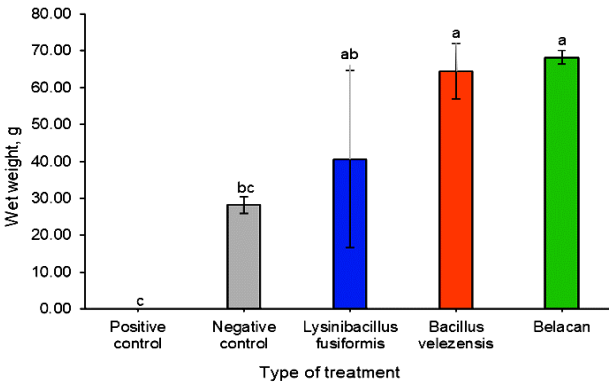


Fig. 5. Wet weight of mushrooms with different treatments. Data were expressed in mean ± standard deviation. Different lowercase letter indicates significant difference ($p<0.05$).

Physical parameters of mushrooms with different treatments

Fig. 6(a) showed pileus width, stipe girth, and stipe length of mushrooms treated with different treatments while **Fig. 6(b)** showed the total number of pins of mushrooms treated with different treatments. *Belacan*, *Lysinibacillus fusiformis* and *Bacillus velezensis* were insignificant in terms of their cap diameter. However, these treatments produced significantly higher outcomes compared to positive and negative controls; indicating that *belacan* and the bacterial treatments may stimulate mycelial growth and colonization of the substrate. Extensive mycelial growth contributed to the development of a robust and productive mushroom fruiting body and larger cap diameters [9].

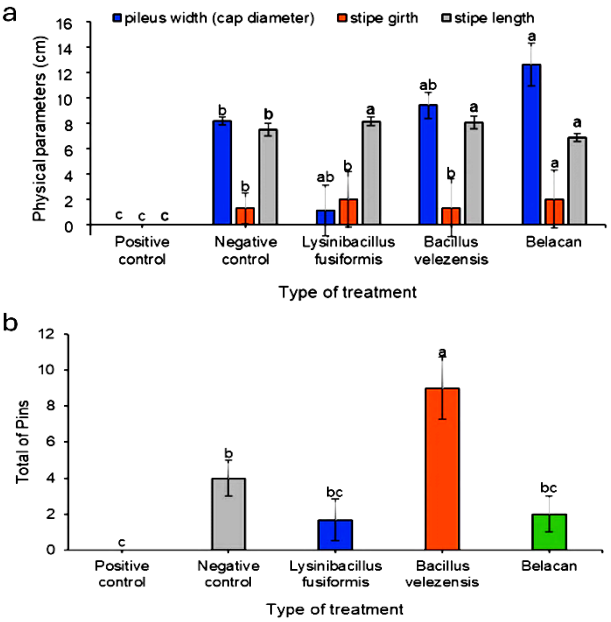


Fig. 1. Different parameters on mushroom measurements (a) Physical parameters; pileus width, stipe girth, stipe girth, and (b) Total of pins. Bars with the same letter are not significantly different ($p<0.05$).

Number of days from complete spawn to first harvest and pinhead of different treatments

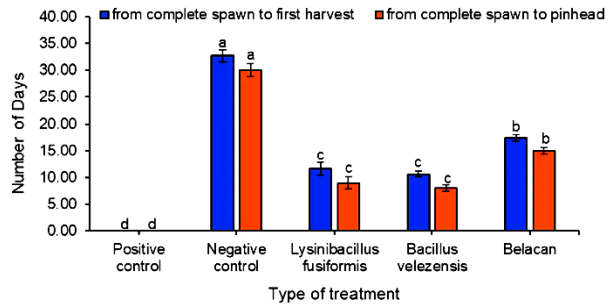


Fig. 7. Number of days from complete spawn to first harvest and pinhead of different types of treatments. *Note.* Bars with the same letter are not significantly different ($p<0.05$).

Fig. 7 depicts the number of days from complete spawn to pinhead formation and first harvest for various treatments. Oyster mushrooms were typically harvested within 1-2 days of pinhead formation. *Lysinibacillus fusiformis* and *Bacillus velezensis* showed statistically higher efficacy compared to *belacan*, positive, and negative controls ($p<0.05$), although they did not significantly differ from each other. This is attributed to their ability to solubilize nutrients in the substrate, thus enhancing nutrient availability for mushroom mycelium, and promoting accelerated mycelial development and subsequent pinhead initiation [10-11].

CONCLUSION

Individual bacterial constituents found in *belacan*, rather than its raw forms, such as *Lysinibacillus fusiformis* and *Bacillus velezensis*, show promise in promoting plant growth, as demonstrated by positive results in in vitro analyses. These analyses include phosphate solubilization, inhibition of *Trichoderma*, and increased levels of Indole-3-acetic acid (IAA). Future research should include potassium and nitrogen testing to further elucidate nutrient availability facilitated by bacteria within *belacan*. Additionally, assessing bioactive compounds in mushrooms would provide valuable insights into any observed increments.

ACKNOWLEDGMENT

This research is funded by Universiti Putra Malaysia Inisiatif Putra Siswazah Grant, with a reference to UPM.RMC.800-2/1/2022/GP-IPS/9740400.

REFERENCES

1. Jaafar A, Mohd Zaini NS, Wan-Mohtar WA, Meor Hussin AS, Abd Rahim MH. Production, organoleptic, and biological activities of belacan (shrimp paste) and pekasam (fermented freshwater fish), the ethnic food from the Malay Archipelago. Sains Malays. 2023;52:1217-30.
2. Mohd Zaini NS, Idris H, Yaacob JS, Wan-Mohtar WA, Putra Samsudin NI, Abdul Sukor AS, Lim EJ, Abd Rahim MH. The potential of fermented food from Southeast Asia as biofertiliser. Horticulturae. 2022;8(2):102.
3. Chandra S, Askari K, Kumari M. Optimization of indole acetic acid production by isolated bacteria from Stevia rebaudiana rhizosphere and its effects on plant growth. J Genet Eng Biotechnol. 2018;16(2):581-6.
4. Srinivasan R, Yandigeri MS, Kashyap S, Alagawadi AR. Effect of salt on survival and P-solubilization potential of phosphate solubilizing microorganisms from salt affected soils. Saudi J Biol Sci. 2012;19(4):427-34.
5. Boiu-Sicuia OA, Toma RC, Diguță CF, Matei F, Cornea CP. In vitro evaluation of some endophytic Bacillus to potentially inhibit grape and grapevine fungal pathogens. Plants. 2023;12(13):2553.
6. Advanced Microbial Techniques in Agriculture, Environment, and Health Management. 2023. Elsevier EBooks.
7. Shi TQ, Peng H, Zeng SY, Ji RY, Shi K, Huang H, Ji XJ. Microbial production of plant hormones: opportunities and challenges. Bioengineered. 2017;8(2):124-8.
8. Verma P, Yadav AN, Khannam KS, Kumar S, Saxena AK, Suman A. Molecular diversity and multifarious plant growth promoting attributes of Bacilli associated with wheat (Triticum aestivum L.) rhizosphere from six diverse agro-ecological zones of India. J Basic Microbiol. 2016;56(1):44-58.
9. Dhakal P, Pokhrel A, Bista A, Shah K, Acharya B, Shrestha J. Growth and yield performance of oyster mushroom (Pleurotus ostreatus) on different substrates. Agriways. 2020;8(1):1-8.
10. Cetin M, Atila F. The potential of bioinoculants in enhancing the mushroom productivity. In: BIO Web of Conferences 2024 (Vol. 85, p. 01049). EDP Sci.
11. Zaini NS, Khudair AJ, Mohsin AZ, Lim EJ, Idris H, Yaacob JS, Rahim MH. Biotransformation of food waste into biofertilisers through composting and anaerobic digestion: a review. Plant Soil Environ. 2023;69.