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RESEARCH ARTICLE

Optimization of Starter Bokashi Bran from Expired Mushroom Blocks Using Central Composite Design

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ABSTRACT - The worldwide market for mushroom cultivation has witnessed substantial growth, particularly in the production of edible mushroom, resulting in significant volumes of agro-industrial waste generated globally. It draws attention to a significant economic and environmental challenge, specifically the storage and disposal of expired mushroom blocks (EMBs) remaining after harvest. Importance of ongoing biotechnological research focused on fungi utilizing various agro-industrial wastes to produce biofertilizer for industrial. The study proposes using EMBs as a substrate for Bokashi fermentation as fertilizer, focusing on the development of starter Bokashi bran. This study identifies and optimize bacterial and fungal growth in Bokashi fermentation using starter Bokashi bran sample from ADA Fresh Farm, Johor Bharu using Response surface methodology (RSM) in Design Experts (DE) software version 7.0. Factors affecting growth for bacterial and fungal were analysed: EMBs content (2 g/ml to 10 g/ml) and fermentation durations (5 days to 9 days). The analysis analysed the highest bacterial and fungal growth in starter Bokashi bran using software predicting maximum growth at 8 days and 4g/ml respectively. Quadratic model was well fitted (R-squared>0.80) with a confidence level higher than 95 % showed that EMBs content and fermentation durations were significant to the bacterial and fungal growth in the starter Bokashi bran. The research demonstrated that EMBs can be utilized as alternative base for starter Bokashi bran that are eco-friendly and sustainable biofertilizer.

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1.0 INTRODUCTION

Due to rising demand brought on by agricultural transformation programs, the mushroom industry has seen tremendous expansion [3]. For every kilogram of mushrooms harvested, the cultivation of mushrooms results in the production of three kilograms of expired mushroom blocks (EMBs), a soil-like material made up of peat, straw, and wheat bran [9]. For the cultivation of mushrooms, agricultural wastes, especially lignocellulosic residues, offer a sustainable resource. The biomass left over after commercial mushroom harvesting is referred to as EMBs, and it has the potential to yield useful items such as animal feed and fertilizers. According to [2], [12], [15] certain mushroom species, such as *Pleurotus sp*., are suitable for fertilizer and ruminant feeding. Using mushroom waste, especially EMBs and mushroom stems, as a biofertilizer is one way to handle this problem. Living microorganisms in biofertilizers increase soil productivity and nutrient availability.

Utilizing composting is an economical and eco-friendly approach to manage mushroom waste. It involves converting agricultural waste disinfectant into usable organic matter through the presence of oxygen, humidity, and temperature [4], [9]. Bokashi composting is a domestic composting method that utilizes an anaerobic process involving organic matter, effective microorganisms (EM), molasses, and water. The Bokashi composting was developed using EM by Professor Teruo Higa in 1982 at University of Ryukyus in Okinawa [9]. The anaerobic process of bokashi fermentation, which uses lactic acid fermentation (LAF), is used to create biofertilizers. By removing oxygen from the surrounding environment, this method encourages bacterial growth while maintaining the quality of the nutrients.

Optimizing individual variables that affect starter Bokashi bran production required conducting multiple experiments separately, leading to heightened time and costs, as well as increased consumption of additional reagents and chemicals. To improve this technique, statistical analysis was applied extensively during the optimization study. Response surface methodology (RSM) involves statistical methods to planning experiments, designing models, evaluating effect variables, and determining the optimal conditions. It is widely used in medium optimization since it establishes and quantifies correlations between various factors fast and with few trials. During the process optimization stage, the Central composite design (CCD) in RSM is especially helpful for fitting second-order quadratic polynomials [5]. Currently, the application of Bokashi fermentation using EMBs for starter Bokashi bran has never been reported. The currently material used for starter Bokashi bran base in the market were either wheat bran, rice bran and castor bean bran [10], [13]. Therefore, this research was added value to expand the knowledge on the production of sustainable biofertilizers using alternatives

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material which is mushroom waste that will help to reduce agriculture waste generation from mushroom cultivation. The findings of this study, which identified optimal fermentation durations and EMBs content to produce bacterial- and fungal-rich Starter Bokashi bran, are vital for consumers aiming to produce biofertilizers. This approach holds significance in mitigating the environmental effects of chemical fertilizers, particularly concerning eutrophication and soil health.

2.0 METHODS AND MATERIAL

2.1 Collection of Expired Mushroom Blocks and Effective Microbe (EM)

Expired mushroom blocks (EMBs) and effective microbes (EM) were collected from ADA Fresh Farm, Batu Pahat, Johor. The sample was deposited in the FTKKP laboratory.

2.2 Preparation of Potato Dextrose Agar (PDA) and Nutrient Agar (NA)

The PDA and NA were purchased from Orioner Hightech at Cyberjaya, Selangor. The PDA was prepared by mixing 39 g of PDA powder in 1 L of distilled water, and NA was prepared by mixing 29 g of NA powder in 1 litre of distilled water. Both solutions were vigorously shaken and then autoclaved for 15 min at 121°C. After being autoclaved, both solutions were poured into the petri dish in the biosafety cabinet. Then, One-third of the petri dish's volume was filled with solution. Lastly, the solutions were left to solidify, and after that, the agar was kept in a chiller for further use. The PDA and NA had final pH of 5.6 ± 0.2 and 6.8 ± 0.2, respectively [6].

2.3 Preliminary Studies 1 and 2 (Fungal and Bacterial Detection)

Conical flask of 250 ml volume was used in the experiment. Firstly, five conical flasks were selected, and each of the flasks was labelled as day 0, day 1, day 2, day 4 and day 7. After that, each conical flask was filled with the expired mushroom block (EMBs) and EM following Starter Bokashi bran ratio that being used by ADA Fresh Farm, Johor ratio (3kg of EMBs: 500 ml of EM) or 6 g/ml. Then, all the conical flasks were closed with a piece of cloth and aluminium foil to create an anaerobic fermentation condition [11]. The flasks were shaken inside the incubator shaker for 5 minutes at 250 rpm and 30 °C before being stored in the incubator at 30°C. The conical flask was taken out from the incubator following its date label and stored inside the freezer at 2°C to stop the fermentation process.

2.4 Experimental Setup for Optimization

As an RSM optimization tool, central composite design (CCD) was used to investigate the influences of independent variables on the fermentation process. Two independent variables were considered to determine optimum condition: EMBs content and fermentation durations. The influences of EMBs content and fermentation durations on the bacterial and fungal growth in the starter Bokashi bran were subsequently analysed. The EMBs content was calculated by dividing the amount (g) of EMBs by the volume (ml) of EM. The amount (g) of EMBs was fixed at 15 g for each trial.

The CCD included 2^n factorial runs, 2^n axial runs where n was experimented input factors and nc was centre runs [8]. The determination of experimental error and result reproducibility relies on the centre points. The model prediction variance remains constant at equidistant points from the design centre, and the selection of axial locations was made to ensure scalability [8]. The total runs of experiments generated by DE software was 13 runs with 5 centre points. The experimental data obtained were studied using Design Expert Version 7.0 software. Table 1 showed the selected factors and it range for the simulation input in the DE. The ranges were selected based on standard ratio used from ADA Fresh Farm, Johor Bharu. The responses were analysed using the Central composite design (CCD) to determine the most contributing factor and interaction between the factors. Table 2 summarizes the data analysis using Central composite design (CCD).

Table 1. Selected factors and their range

Factors	Range
A – EMBs content	2 – 10 g/ml
B – Fermentation durations	5 – 9 days

2.5 Serial Dilution

A recognizable or unknown entity, such as a solute or organism can be systematically reduced via serial dilution, a process that involves repeatedly re-suspension of an original solution (the solute) into specified volume of a liquid diluent (blanks) [6]. These blanks may vary in composition, but they usually consist of 0.45% saline. The experimenters can choose the volume of each diluent, but it is typically a multiple of 10 to allow for logarithmic reduction of the sample [6].

In this study, 90% saline solution was used to produce dilution liquid, and 9 ml was poured into each test tube. At first, 1 gram of Bokashi was transferred into test tubes that contained 9 ml of saline solution. The broth was then vigorously shaken, and then 1 ml of the solution was transferred into another test tube containing 9 ml of saline solution. The steps were repeated four times.

Table 2. Data analysis using central composite design

Runs	Factors	
	A- EMBS content (g/ml)	B- Fermentation durations (Days)
1	4.00	6.00
2	8.00	6.00
3	4.00	8.00
4	8.00	8.00
5	2.00	7.00
6	10.00	7.00
7	6.00	5.00
8	6.00	9.00
9	6.00	7.00
10	6.00	7.00
11	6.00	7.00
12	6.00	7.00
13	6.00	7.00

3.0 RESULTS AND DISCUSSION

3.1 Preliminary Studies of Starter Bokashi Bran Ratio

Preliminary studies were conducted to test the starter Bokashi bran ratio used at ADA Fresh Farm, Johor. Figure 1 showed data for plate colony count of bacterial and fungal in CFU/ml unit. The peak plate colony counts for fungi occurred on day 7, reaching 6.68 x 10^7 CFU/ml, while for bacteria, it was observed on day 4, totaling 4.81 x 10^7 CFU/ml, respectively. These results suggest a beneficial influence of EMBs on the growth of both bacterial and fungal. EMBs demonstrated potential as an alternative substrate for fostering beneficial microbial activity essential for composting. This suitability stems from its composition, containing lignocellulosic materials known for their role in mushroom substrate preparation along with fermentable sugars [14], [16], and [18]. These components provide nourishment for the rapid proliferation of beneficial fungi and bacteria. As microbial populations increase, the composting process could significantly accelerate.

According to [19], increasing of beneficial bacterial consortium greatly increased the amount of organic matter in the soil. Additionally, it was discovered that the combination of microbes in organic fertilizer had the highest soil content of available phosphorus, potassium, and total and available nitrogen, which later affected the growth stage (full fruit stage and harvesting time) [17], [19]. Thus, using phosphate-solubilizing microorganisms as soluble phosphorus suppliers in fertilizers offers a viable approach from both an economic and environmental standpoint.

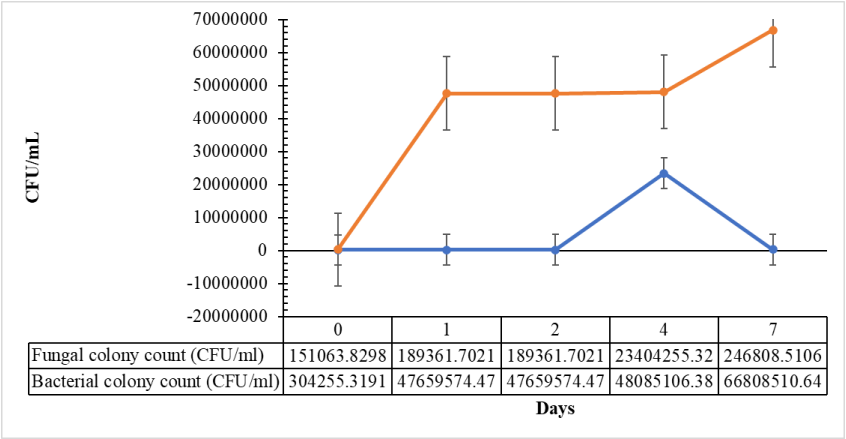

Figure 1. Fungal and bacterial colony count in starter Bokashi bran

3.2 Optimization of Starter Bokashi Bran (Fungal and Bacterial Count)

Table 3 showed data that was analyzed using Design Expert Software to achieve the optimized condition of the starter Bokashi fermentation. ANOVA was conducted to determine the model coefficient and assess the significance of the chosen variables. In this research, ANOVA was utilized to determine the variables and their interactions relevance to the studies. The statistical significance of the regression equation was determined through F-values, while the significance of individual coefficients was assessed using p-values [20]. A p-value below 0.05 indicates the significance of model terms [20]. The lack of fit analysis evaluated the accuracy of model fitting by comparing actual and predicted data [20]. Unlike the whole-model test which assesses the significance of all terms in the model, the lack of fit test examines the significance of any excluded elements. The A significant lack of fit suggests well-replicated runs with minimal variance [11]. Table 4

and table 5 showed the ANOVA for fungal and bacterial model. The F-value of the model fungal and bacterial is 0.0048 and 0.0002 respectively, suggesting that the model for both fungal and bacterial is significant, with only a 0.408 % and 0.02% chance that the value could be attributed to noise, respectively.

In optimization, the selected mathematical model generally targeted was the quadratic model. However, in the analysis, the fungal detection showed that linear vs mean was suggested by the software, while the bacterial detection showed that a quadratic model was proposed. For the sake of the analysis, the fungal detection model was manually selected as a quadratic model. Then, the selected mathematical model must be significant so that it can be used for the optimization analysis. The standard deviation (R^2) of the model also must be greater than 0.8 for the bio-process analysis. Each quadratic model is significant in the experiments and had a standard deviation greater than 0.8 (0.8733 for fungal detection and 0.9539 for bacterial detection).

The correlation between the main primary variables with fungal and bacterial CFU can be determined by the quadratic equations in coded terms as presented by equation 1 and equation 2. The equation models showed correlating the interactions input and output variables with coded A representing EMBs content (g/ml) and B representing fermentation durations (days). AB represents the interaction between variables A and B.

$$Fungal\ CFU = 4.65 - 0.99A + 0.54B + 0.12AB + 0.23A^2 - 0.12B^2 \quad (1)$$

$$Bacterial\ CFU = 6.46 - 0.99A + 0.49B - 0.69AB - 0.19A^2 - 0.48B^2 \quad (2)$$

Table 3. Centre Composite Design Table

Std	Run	Factor 1 (A): EMBS Content (g/ml)	Factor 2 (B): Fermentation Durations (Days)	Response 1: Fungi CFU (CFU/ml x 10^7)	Response 2: Bacterial CFU (CFU/ml x 10^7)
1	4	4	6	5.45	5.54
2	11	8	6	3.82	4.8
3	7	4	8	6.55	7.56
4	8	8	8	5.42	4.39
5	13	2	7	7.56	7.79
6	5	10	7	3.02	3.79
7	6	6	5	2.93	3.61
8	10	6	9	4.83	5.72
9	9	6	7	4.27	5.81
10	1	6	7	4.53	6.64
11	3	6	7	4.27	6.61
12	12	6	7	4.62	6.79
13	2	6	7	4.44	6.87

Table 4. Mathematical Model of Fungal Detection

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob > F)	
Model	17.36	5	3.47	9.65	0.0048	significant
A-EM concentration	11.68	1	11.68	32.48	0.0007	
B-Duration Fermentation	3.52	1	3.52	9.79	0.0166	
AB	0.06	1	0.06	0.17	0.6893	
A^2	1.21	1	1.21	3.37	0.1092	
B^2	0.34	1	0.34	0.96	0.3603	
Residual	2.52	7	0.36			
Lack of Fit	2.42	3	0.81	33.16	0.0028	significant
Pure Error	0.10	4	0.02			
Cor Total	19.87	12				

R^2 = 0. 8733. *P-values exceeding 0.05 suggest that the model terms lack significance.

In optimization, the contour plot of the factor must be in the center of the data analyzed; otherwise, the optimization point has not yet been defined. If the optimization point was not yet defined, the experiment must be repeated with a different range for the selected factors. In the analysis, both bacterial and fungal detection showed that the optimization point was yet to be defined. Meanwhile, the data is sufficient to predict the interactions between independent variables with the main variables of the experiments. Figure 2 and figure 3 showed that both fungal and bacterial contour models

showed that the optimum conditions are within the ranges of the studies and the data is sufficient to predict the optimum conditions. The data showed positive interaction effect between independent variables and main variables that was enough to be used for the research findings.

Table 5. Mathematical Model of Bacterial Detection

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob > F)	
Model	21.39	5	4.28	28.99	0.0002	significant
A-EM concentration	11.82	1	11.82	80.11	< 0.0001	
B-Duration Fermentation	2.83	1	2.83	19.20	0.0032	
AB	1.48	1	1.48	10.01	0.0159	
A^2	0.86	1	0.86	5.84	0.0463	
B^2	5.17	1	5.17	35.06	0.0006	
Residual	1.03	7	0.15			
Lack of Fit	0.31	3	0.10	0.58	0.6578	significant
Pure Error	0.72	4	0.18			
Cor Total	22.42	12				

R^2 = 0.9539. * P-values exceeding 0.05 suggest that the model terms lack significance.

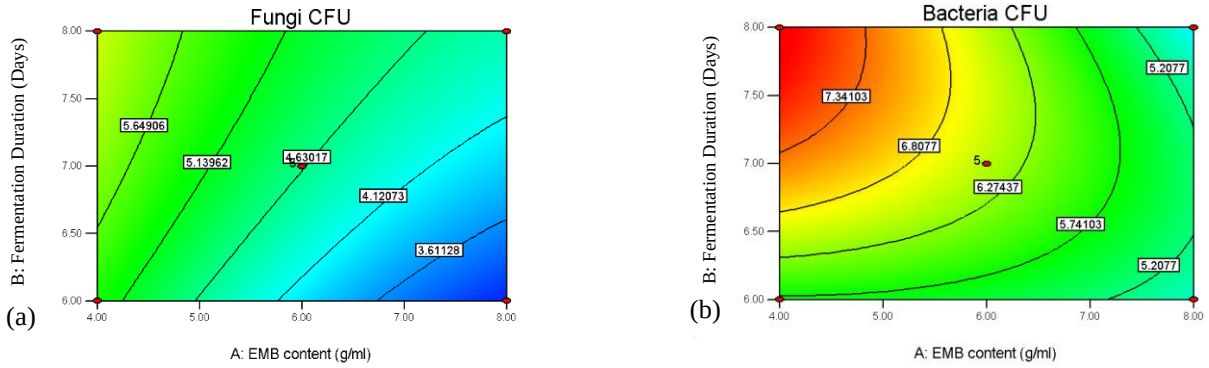

Figure 2. Optimum contour plot for (a) fungal detection and (b) bacterial detection

In these experiments, the fungal and bacterial detection must be maximized so that the bacterial and fungal growth in starter Bokashi bran is optimum and improvised. So, the selected factors were kept within the range while bacterial and fungal detection were being maximized in the simulation.

3.3 Optimum Condition Analysis

Based on the simulation results, the optimal conditions for the starter Bokashi bran involve achieving maximum bacterial and fungal growth through a duration of 8 days and an EMBs concentration of 4 g/ml. The expected fungal and bacterial growth were 6.16 x 10^7 CFU/ml and 7.87 x 10^7 CFU/ml. The validation of the optimal conditions was conducted by comparing them with the preliminary studies of the study. This was because the preliminary study data originated from ADA Fresh Farm, Johor Bharu, aligning with actual data and customer needs for this study. As part of the validation process, the performance of the optimized solution was compared with real-world data to verify its accuracy and suitability. Consequently, the preliminary data provided adequate insight into the current performance of the starter Bokashi bran used in ADA Fresh Farm, Johor Bharu serving as the baseline for validating the optimal conditions. The expected growth of fungi and bacteria was estimated to be 6.16 x 10^7 CFU/ml and 7.87 x 10^7 CFU/ml, respectively. Due to resource limitations and the preliminary alignment with customer needs, the data proved suitable for replacing the validation process in this study. Therefore, in validating the optimal conditions, the preliminary data acted as a reference point, reflecting the actual conditions observed at ADA Fresh Farm, Johor Bharu. A comparison with previous studies using starter Bokashi bran ratios from ADA Fresh Farm, Johor Bharu revealed a 25% improvement in bacterial growth, indicating that the optimized conditions can yield a higher quality of starter Bokashi bran in a shorter period as showed in figure 4. This improvement not only eases the economic burden on companies producing biofertilizers but also reduces agricultural waste volume in mushroom cultivation.

The microbial counts were observed to positively increase with fermentation durations was also reported in [1], [21]. The findings suggested the importance of carefully optimized fermentation durations for achieving a harmonious growth balance between fungal and bacterial, ensuring the well-balanced and effective biofertilizers. The dynamics interaction between these two microbial groups, with optimized fermentation durations would be supporting a balance and diverse

microbial consortium beneficial for plant growth. Meanwhile, the EMBs contents influenced the efficiency of microbial groups according to [7]. The study found a direct correlation between lignocellulose concentrations and growth of microbial consortium that affect degradation of the lignocellulose material. It concludes that the importance of optimized balance fermentation durations and EMBs contents in supporting effective organic matter decomposition for the biofertilizer production as nitrogen (N), phosphorus (P) and potassium (K) concentrations directly increase in the decomposition process.

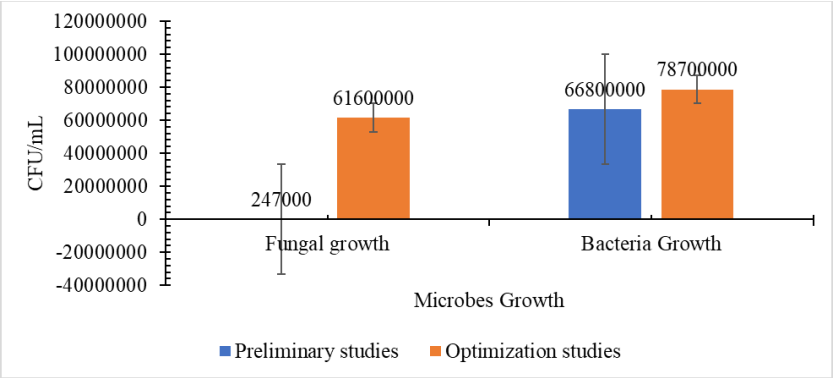

Figure 3. Preliminary vs Optimization

4.0 CONCLUSION

The influence of fermentation days and EMBs content on the fungal and bacterial growth was determined in this study, with both factors being significant for starter Bokashi bran production. It was also determined that EMBs was suitable for alternatives based for the starter Bokashi bran production. The optimum starter Bokashi bran production was obtained at 4 g/ml EMBs contents and 8 days of fermentation days with fungal and bacterial value at their peak growth value of 6.16×10^7 CFU/ml and 7.87×10^7 CFU/ml, respectively. The findings of this study could be used to increase high grade of starter Bokashi bran for the consumers to produce biofertilizers that could help economic growth and sustainable production.

5.0 CONFLICT OF INTEREST

The authors declare that they have no identifiable competing financial interests or personal relationships that could have appeared to impact the study reported in this paper.

6.0 AUTHORS CONTRIBUTION

A. S. Baharudin: Investigation, methodology, Formal analysis, Data curation, writing – original draft, Visualisation.

N. Zainol: Conceptualisation, Writing – review and editing, Project administration, Supervision, Funding acquisition.

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