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Plackett-Burman Design of Low-Cost Culture Medium for Biomass Production of Probiotic *Bacillus licheniformis* CCASU-2024-65 and *Bacillus subtilis* (CCASU-2024-67) Strains

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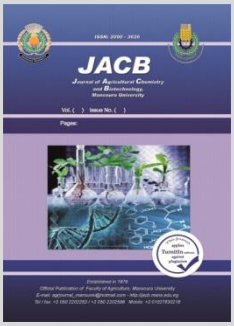

ABSTRACT

The management of agro-waste and its conversion into useable products using biotechnological applications in agriculture is garnering significant interest. *Bacillus* strains are commonly used in poultry cultures as probiotics or single-cell proteins. However, their high production costs and low biomass yields limit their use as microbial proteins in animal feeds. Biomass production of *Bacillus licheniformis* and *Bacillus subtilis* is a growing area of interest in agriculture due to its nutritional benefits. This study evaluated various agro-waste culture media for biomass production of these probiotics, utilizing banana, pea, rice husks, and potato peels. The highest biomass production was achieved by *Bacillus licheniformis* (CCASU-2024-65) at 1.97 g/L with banana peel extract medium, while *Bacillus subtilis* (CCASU-2024-67) recorded high biomass production of 1.5 g/L with potato peel extract medium. The statistical Plackett-Burman Design was used to screen nutrient components and culture conditions for these probiotics. The maximum biomass production of *Bacillus licheniformis* was 2.7 g/l, achieved at run number 8, which had ideal conditions comprising an initial pH of 6, 50 g/L of banana peel extract, 50 g/L of pea peel extract, a 10% inoculation ratio, and a fermentation duration of 48 hours for biomass generation. *Bacillus subtilis* produced 3.52 g/l output at run number 12, which had output occurred at run number 12, which comprised 50 g/L potato peel extract and 50 g/L pea peel extract concentration, an initial pH of 7, a 10% inoculum size, and a fermentation duration of 48 hours.

Keywords: Ago wastes, *Bacillus*, Biomass productions and Probiotics

INTRODUCTION

Beneficial microorganisms known as probiotics perform a crucial function in maintaining the general health of their host. Among these, the *Bacillus* genus, comprising Gram-positive bacteria, is widely utilized as probiotics for both humans and animals. These probiotics are available in various commercial formulations. *Bacillus licheniformis* and *Bacillus subtilis* are notable strains employed as probiotics (Romo *et al*., 2021). These species can be used as probiotics because of their capacity to create enzymes and secondary metabolites that are recognized for inhibiting the growth of harmful microbes (Rhayat *et al*., 2019; Lim *et al*., 2021; Ali *et al*., 2024). They are extensively used as probiotics in veterinary medicine (Inatsu *et al*., 2006; Chen & Yu, 2020). Reports of infections caused by *Bacillus* species have engendered skepticism about their application as probiotics, owing to the potential generation of toxins linked to diarrheal diseases and their possible antibiotic resistance (Sankararaman & Velayuthan 2013).

The optimal nutritional status of microorganisms, including nitrogen compounds, carbohydrates, micro- and macroelements, and other biologically active substances, during cultivation, is contingent upon the meticulous selection of nutrient media according to their properties (Rakhmetov *et al*., 2023). Deep cultivation is the most appropriate strategy for generating bacterial biomass for biopreparations (Tihonovich & Kruglov, 2006; Kalenska *et al*., 2023). Nonetheless, these culture mediums are costly due to their use of nitrogen sources such as beef, peptones, yeast extract, and meat extract, which may account for 30–40% of production expenses (Galante *et al*., 2023). An alternative to commercial media is the utilization of agro-industrial by-products (Patel & Shukla 2017; Galanakis 2019). Which can serve a dual purpose: providing nutrients for biomass production and functioning as a protective wall material in drying processes for probiotics

The utilization of agricultural waste in culture media has arisen as a circular economy approach to address substantial waste volumes. This waste can supply nutrients, particularly the organic carbon essential for microbial development (de Medeiros *et al*., 2020 & Santos *et al*., 2021). By-products from the agro-industrial sectors of fruit, brewing, and dairy industries have been examined for their potential to enhance the biological activity of bacteria and generate bioactive chemicals (Bartkiene *et al*., 2019 & Mathias *et al*., 2015).

Plackett–Burman design (PBD) is an efficient methodology for identifying important components, optimizing time, and assuring precise parameter definition (Abdel-Fattah *et al*., 2005). This study delved into the practical application prospects of biomass production for *Bacillus licheniformis* CCASU-2024-65 and *Bacillus subtilis* (CCASU-2024-67) by using agricultural wastes as medium to large-scale, low-cost output. The medium ingredients and culture conditions that have a major impact on biomass output were determined using the Plackett-Burman design (PBD). The medium ingredients and culture conditions that have a major impact on biomass output were determined using the Plackett-Burman design (PBD).

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MATERIALS AND METHODS

Microorganisms

The bacterial strains *Bacillus licheniformis* (CCASU-2024-65) and *Bacillus subtilis* (CCASU-2024-67) under accession numbers ON054301 and ON054303, respectively (Ali *et al*., 2024) were obtained from the Microbiology Department of the Faculty of Agriculture at Beni Suef University. The cells suspension was cultures on nutrient agar slants and preserved in 40% glycerol and stored at −20°C.

The process of cells culture

A single-cell culture was employed to inoculate 100 mL of nutrient broth in a 250-mL flask, which was incubated in a rotating shaker at 35°C and 150 rpm for twenty-four hours

Wastes material preparation

Large quantities of potato, banana, pea and rice peels were collected from homes and fields. The peels were washed to remove dust, dried for a week at room temperature, and crushed to produce extracts. Five grams of crushed peels were dissolved in 100 ml of tap water boiled in a water bath at 100 ^{o} C for 30 minutes. The extracts were filtrated, used as a whole medium, and tested for strain growth. Four peel extracts were employed individually for biomass production.

Culture Conditions

The study used culture parameters including temperature (35°C), pH (7), agitation (150 rpm), and inoculum volume (5% v/v). The strain's cultivation time was determined to be 24 hours after a trial.

Plackett-Burman design (PBD) screening of culture conditions and medium components

The Plackett-Burman Design (PBD), employing a first-degree model [Equation 1], was implemented to evaluate the comparative importance of the medium constituents and environment variables on bacterial cells generation (Plackett and Burman, 1946).

$$Y = \beta_0 + \Sigma \beta_i X_i \quad \textbf{[Equation 1]}$$

The model intercept is denoted by β0, Y represents the response variable, the coefficient of linearity is denoted by βi, and Xi depicts the magnitude of the factor that is independent in this context. The design specifies that the all quantity of tested trials is n + 1, where n is the amount of parameters. Nutritional elements, comprising potato, banana, pea, and rice extracts, were assessed in conjunction with cultural requirements, including pH, inoculum volume, and fermentation duration, over 12 experimental trials. The amount ranges of the media components and the growth environments employed in the PBD were determined using an initial one-factor-at-a-time (OFAT) methodology. The effect variable was a yield of cells (g/L) measured at a temperature of 35 °C and an agitation rate of 150 rpm. Any element in the PBD was examined at two levels: low (-) and high (+) (Table 1). Centre point (0) replications were performed in three separate runs.

Table 1. Coded and uncoded values of experimental variables used in Plackett- Burman design.

Factor	Name	Coded levels	
		Low level (-)	High level(+)
A	pH	6	7
B	Inoculum size (%)	5	10
C	Fermentation time (h)	24	48
D	Rice peel(g/L)	0	50
E	Potato peel(g/L)	0	50
F	Pea peel(g/L)	0	50
G	Banana peel(g /1L)	0	50

Measurement of Biomass Growth

Following the fermentation period, the biomass produced by *Bacillus licheniformis* (CCASU-2024-65) and *Bacillus subtilis* (CCASU-2024-67) cultivated in an experimental medium was assessed. The dry mass of bacterial growth was determined by centrifuging 10 mL of culture at 5000 xg for 20 minutes, followed by desiccation at 80°C for 24h, and measuring the resulting dried-cell biomass in g/l.

The statistical testing

Statistics design and evaluation were performed utilizing Design Expert 12. The models were subjected to statistical analysis by analysis of variance (ANOVA). The significance of the regression coefficient of determination (R^2) in statistics, evaluated by the F-test, was utilized to examine the statistical validity of the polynomial model equations. The significance threshold significance level for all statistical tests was established at 0.05. The student t-test evaluated the statistically significant value of the regression coefficients. The Pareto chart in PBD was employed to discern unimportant variables at a significance level of p = 0.05, elucidating the primary and interacting impact of the various independent variables on the yield of cells of *Bacillus licheniformis* (CCASU-2024-65) and *Bacillus subtilis* (CCASU-2024-67).

RESULTS AND DISCUSSION

Banana peels account for around 3.5 million tonnes of garbage each year (Ewing-Chow 2022). Banana peels are an economical and renewable organic waste material. Their abundance of vital nutrients, such as carbohydrates, phenolic compounds, proteins, vitamins, and macro- or micronutrients, may facilitate microbial development (Singh *et al*., 2023). The peel of the banana an organic waste is rich in carbohydrates and other essential ingredients that promote microbial growth (Yasin *et al*., 2019).

The data illustrated in Figure (1) indicated that the peak biomass production by *Bacillus licheniformis* (CCASU-2024-65) utilizing banana peel extract medium was 1.97 g/L after 24 hours at pH 7, with a productivity of 0.08 $gL^{-1}h^{-1}$.

Potato peel, a residue of the food processing sector, function as a cost-effective, valuable, and readily available raw material for the synthesis of compounds of economic significance, value augmentation, and product extraction, encompassing dietary fiber, biopolymers, natural antioxidants, and natural food additives (Chiellini *et al*., 2004). Industry manufacturing yields around 70,000 to 140,000 tons of peels annually globally (Hang 2009). According to Jafrizal *et al*. (2017), potatoes generate 15% of waste; thus, based on 2021 potato production estimates, the potential for potato peel waste amounts to 0.2 million tons. The current utilization remains minimal, mostly employed as a compost additive. The chemical constituents in potato peels hold significant relevance. The composition of potato peels consists of 68.7% carbon, 1.3% nitrogen, and the remainder is oxygen. (Widyastuti & Kunsah, 2017).

The research findings verified the economic significance of potato banana peels where the greatest biomass output by *Bacillus subtilis* (CCASU-2024-67) utilizing potato peel extract medium was 1.5 g/L after 24 hours at pH 7, with a productivity of 0.75 $gL^{-1}h^{-1}$. The previous data indicated that using waste media (banana and potato peel extract) enhanced biomass production by 1.5 and 1.2 times, respectively, compared to nutrient media for

Bacillus licheniformis (CCASU-2024-65) and *Bacillus subtilis* (CCASU-2024-67).

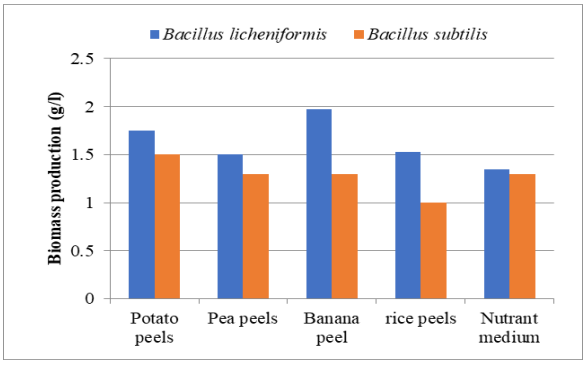

Figure 1. Biomass production of *Bacillus licheniformis* (CCASU-2024-65) and *Bacillus subtilis* (CCASU-2024-67) using different peel extracts.

These results confirm the existing evidence in Jadhav *et al*., 2013 studies were primarily focused on utilizing potato and banana peel materials, both alone and in conjunction, for the production of amylase, an enzyme of significant commercial relevance. The amylase yield from the fermentation substrate was measured at 0.7 units/ml for *Aspergillus niger* and 0.8 units/ml for *Bacillus subtilis*, both identified as amylase producers. One isolate designated BS9, yielded a maximal production of 1.55 units/ml after 72 hours. Nonetheless, PB1 produced unexpected conclusions within just 24 hours, delivering 1.36 units/ml. It also supports what has been shown in Henshaw's (2024) investigation of amylase synthesis from *Bacillus subtilis* IMD34. Extracellular amylase synthesis was conducted in a basal medium using the submerged fermentation approach. The production medium containing 10% potato peels at pH 6.8 and 35 °C exhibited superior amylase activity (720 U/mL) compared to other substrates, including banana and yam peels.

Evaluation of Important Nutrient Elements Applying Plackett-Burman Design

The Plackett-Burman design is an essential instrument for evaluating the influence of variables on the final result (Cavazzuti 2013). The PBD has been applied to determine the significant variables influencing biomass production. Data in Figure 2 showed the biomass yield variability throughout the 12 experimental runs by *Bacillus licheniformis* (CCASU-2024-65) and *Bacillus subtilis* (CCASU-2024-67).

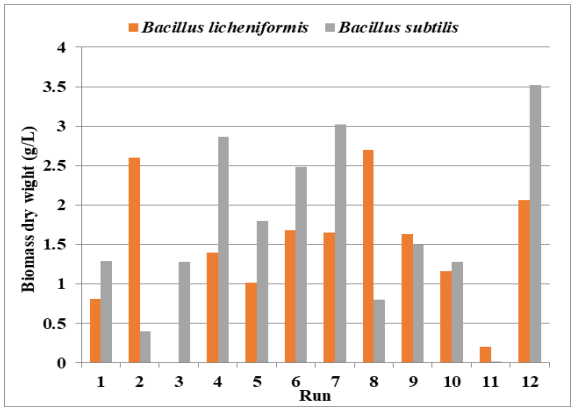

Figure 2. Responses of the Plackett - Burman Design (PBD) biomass production of *Bacillus licheniformis* (CCASU-2024-65) and *Bacillus subtilis* (CCASU-2024-67) using different peel extracts.

Seven distinct variables, encompassing medium composition (rice, potato, pea peels, and banana peels) and cultural conditions such as starting pH, inoculum size, and fermentation duration, were examined (Table 2) for *Bacillus licheniformis* (CCASU-2024-65) and *Bacillus subtilis* (CCASU-2024-67).

Table 2. Design and responses of the Plackett - Burman Design (PBD) biomass production of *Bacillus licheniformis* (CCASU-2024-65) and *Bacillus subtilis* (CCASU-2024-67).

Run	A	B	C	D	E	F	G	Biomass yield	
								B. licheniformis	*B. subtilis*
1	+	-	+	+	-	+	-	0.810	1.29
2	-	+	+	+	-	+	+	2.60	0.400
3	+	+	-	+	-	-	-	0.0100	1.28
4	-	+	+	-	+	-	-	1.40	2.86
5	-	-	-	+	+	+	-	1.02	1.80
6	+	-	+	-	-	-	+	1.68	2.48
7	-	-	+	+	+	-	+	1.65	3.02
8	-	+	-	-	-	+	+	2.70	0.800
9	+	+	-	+	+	-	+	1.63	1.49
10	+	-	-	-	+	+	+	1.16	1.28
11	-	-	-	-	+	-	-	0.200	0.0200
12	+	+	+	-	+	+	-	2.06	3.52

Abundant pea waste generated during industrial processing leads to substantial environmental problems and the potential release of hazardous gasses (Vilariño *et al*., 2017; Tassoni *et al*., 2020).

Unsustainable waste disposal may incur significant economic expenses due to its direct effect on industrial profitability. Malenica & Bhat (2020). Pea peel waste, constituting around 30–40% of the total pea weight, is accessible in bulk at no cost. (Vilariño *et al*., 2017). Consequently, numerous methods are necessary to transform these wastes into valuable goods with elevated nutritional content. These methods involve repurposing pea peel waste as animal feed and utilizing their bioactive chemicals as natural additives in food, cosmetics, and pharmaceutical applications.

Figure 2 indicate that *Bacillus licheniformis* (CCASU-2024-65) exhibited significant variability in biomass production, ranging from 2.7 to 0.2 g/l, likely attributable to interactions between the studied variables. Maximum biomass generation of 2.7 and 2.0 g/l was attained in runs number 8 and 2, respectively. Run 8 possesses the ideal circumstances for biomass generation, comprising an initial pH of 6, 50 g/L of banana peel extract, 50 g/L of pea peel extract, a 10% inoculation ratio, and a fermentation duration of 48 hours. The biomass production of *Bacillus subtilis* varied from 0.02 to 3.52 g/l. Maximum biomass output occurred at run number 12, which comprised 50 g/L potato peel extract and 50 g/L pea peel extract concentration, an initial pH of 7, a 10% inoculum size, and a fermentation duration of 48 hours. The minimal biomass production was recorded in run number 11.

In the PBD model, the evaluated components were subsequently aligned with the multifactorial linear regression equation, anticipating a *P* value of <0.05. Factors with a *P* value <0.05 exhibit a direct relationship to activity, whereas those with a *P* value >0.05 do not demonstrate a linear correlation. Analysis of variance (ANOVA) (Table 3) using The Fisher test was employed to measure the influence of independent factors on the response, with significant results determined by a *p*-

value < 0.05. The model F-value of 63.5 indicates that the model is significant for biomass production and *p*-value was 0.000617. A smaller *p*-value signifies the substantial relevance of the associated coefficient (Tanyildizi *et al*., 2005).

Seven distinct variables, encompassing medium composition (rice, potato, pea peels, and banana peels) and cultural conditions such as starting pH, inoculum size, and fermentation duration, were examined (Table 2) for *Bacillus licheniformis* (CCASU-2024-65) and *Bacillus subtilis* (CCASU-2024-67).

The analyzed results suggests that out of the seven different variables initial pH, inoculum size potato peels , pea peels and banana peels extract significantly affected biomass production by *Bacillus licheniformis* (CCASU-2024-65). The *p*-values of these significant variables ranged from 0.000145to 0.0146 (Figure 3)

In case of *Bacillus subtilis* (CCASU-2024-67), the model F-value of 92.2 implies that the model is significant for biomass production. *P*-value was 0.0108. The analyzed data indicated that only 4 variables including, inoculum size, potato peels, pea peels and rice peels significantly affected biomass production, having *p*-values ranging from 0.00458 to 0.0330 (Figure 4).

The coefficients of determination (R^2) were 0.991 and 0.998 which means that 99% of the model accounted for the complete variation For *Bacillus licheniformis* (CCASU-2024-65) and *Bacillus subtilis* (CCASU-2024-67) (Tables 3&4).

Table 3. Analysis of variance for the experimental results of the Plackett - Burman Design (PBD) biomass production of *Bacillus licheniformis* (CCASU-2024-65).

Source	SS	df	MS	F-value	*p*-value	
Model	7.61	7	1.09	63.5	0.000617	significant
A-PH	0.291	1	0.291	17.0	0.0146	
B-inoculuim size	1.79	1	1.79	105.	0.000515	
C-fermentation time	1.60	1	1.60	93.2	0.000645	
E-potato peel	0.294	1	0.294	17.2	0.0143	
F-pea peel	0.647	1	0.647	37.8	0.00355	
G-banana peel	3.42	1	3.42	200.	0.000145	
AC	0.862	1	0.862	50.3	0.00209	
Residual	0.0685	4	0.0171			
Cor Total	7.68	11				
R^2=0.991						
Std. Dev.=0.131						
C.V. %= 9.28						
Mean=1.41						

DF: degree of freedom; SS: Sum of squares; MS: mean square.

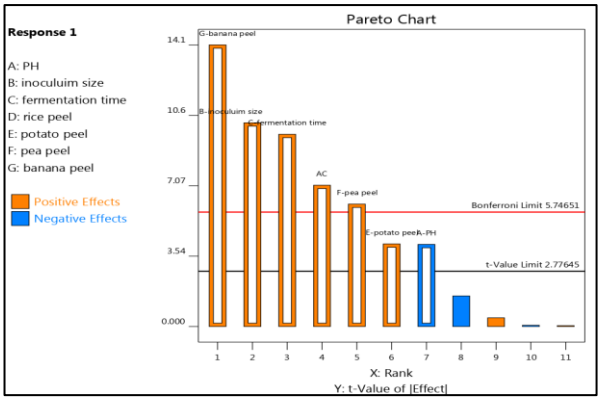

Figure 3. The extent of positive and negative effects of the factors on biomass production of *Bacillus licheniformis* (CCASU-2024-65).

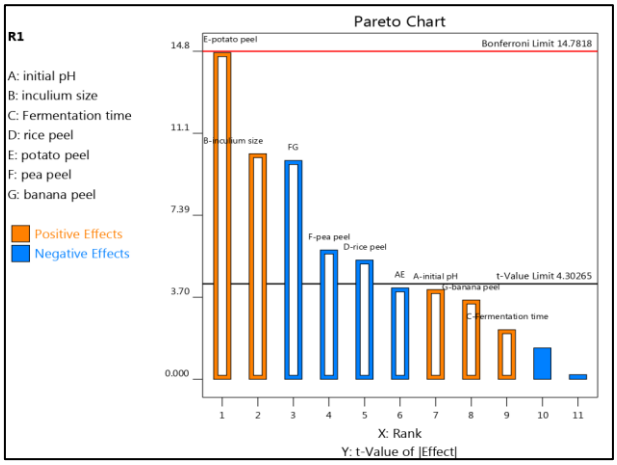

Figure 4. The extent of positive and negative effects of the factors on biomass production of *Bacillus subtilis* (CCASU-2024-67).

Table 4. Analysis of variance for the experimental results of the Plackett - Burman Design (PBD) biomass production of *Bacillus subtilis* (CCASU-2024-67).

Source	SS	df	MS	F-value	*p*-value	
Model	12.9	9	1.43	92.2	0.0108	significant
A-initial pH	0.253	1	0.253	16.3	0.0561	
B-inculium size	1.60	1	1.60	103.	0.00954	
C-Fermentation time	0.0771	1	0.0771	4.97	0.155	
D-rice peel	0.447	1	0.447	28.9	0.0330	
E-potato peel	3.36	1	3.36	217.	0.00458	
F-pea peel	0.526	1	0.526	33.9	0.0282	
G-banana peel	0.198	1	0.198	12.8	0.0703	
AE	0.263	1	0.263	16.9	0.0543	
FG	1.51	1	1.51	97.2	0.0101	
Residual	0.0310	2	0.0155			
Cor Total	12.9	11				
R^2=0.998						
Std. Dev.= 0.125						
C.V. %=7.38						
Mean=1.69						

DF: degree of freedom; SS: Sum of squares; MS: mean square.

This denotes an adequate depiction of the method models and a strong link between the experimental and predicted values for the two strains. By using Design-Expert version 12, the equation obtained for PBD (first-order model) of *Bacillus licheniformis* (CCASU-2024-65) was as follows:

$Y_{\text{biomass production}}$=+11.5 -2.08* initial pH +0.178*inoculum size +0.289*fermentation time+0.0715*Potato peels+0.0965*Pea peel extract +0.246 Banana peels extract (Equation 1)

Whereas, that of *Bacillus subtilis* (CCASU-2024-67) was as follows:

$Y_{\text{biomass production}}$= -5.70+0.747* initial pH+0.201* inoculum size +0.221*fermentation time -0.0971*rice peels extract+0.144*pea peels extract +*0.315 banana peels extract (Equation 2)

Where Y is the predicted biomass production response. From previous results, it could be suggested that the significant factors identified by PBD technique.

In a similar research studies study, Blibech *et al.* (2020) surveyed *Bacillus subtilis*, which produces a previously identified extremely thermostable β-mannanase identified as a possible probiotic candidate for use as a nutritional additive in the poultry industry. The researchers improved fermentation conditions and enhanced enzyme titer by utilizing experimental designs and valorizing agro-industrial wastes. Employing the Plackett–Burman design, a

total of 14 culture factors were assessed in submerged fermentation for their impact on β-mannanase synthesis. Locust bean gum (LBG), soya meal, temperature, and inoculum volume were optimized using a response surface approach with a Box–Behnken design. Subsequently, to reduce the cost of enzyme production, the impact of partially substituting LBG (1 $g\,L^{-1}$) with agro-industrial wastes was examined using a Taguchi design.

CONCLUSION

The innovation of this study is in the economic formula for nutritional media designed for probiotic biomass production. The study designs a media based on agro-industrial by-products to cultivate biomass of *Bacillus licheniformis* and *Bacillus subtilis*. The results indicated that this agro-industrial by-product culture medium serves as a cost-efficient option for the production of probiotic bacteria for animal feed supplements. Thereby fulfilling one of the goals of sustainable development. The impact of several environmental conditions on bacterial development was examined using the Plackett-Burman Design. This has demonstrated efficacy in augmenting bacterial biomass. The article additionally advises doing tests on animal feeding to assess the effectiveness of these bacteria

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تصميم بلاكيت-بورمان لبيئه غذائيه منخفضه التكلفة لإنتاج الكتلة الحيوية من سلالات البروبيوتيك *Bacillus licheniformis* CCASU-2024-65 و *Bacillus subtilis* CCASU-2024-67

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الملخص

تحظى إدارة النفايات الزراعية وتحويلها إلى منتجات قابلة للاستخدام باستخدام التطبيقات التكنولوجية الحيوية في الزراعة باهتمام كبير. تُستخدم سلالات *Bacillus* بشكل شائع في مزارع الدواجن كبروبيوتيك أو بروتينات وحيدة الخلية. ومع ذلك، فإن تكاليف إنتاجها المرتفعة وعائدات الكتلة الحيوية المنخفضة تحد من استخدامها كبروتينات ميكروبية في أعلاف الحيوانات. يعد إنتاج الكتلة الحيوية من *Bacillus licheniformis* و*Bacillus subtilis* مجالًا متزايد الاهتمام في الزراعة بسبب فوائدها الغذائية. قامت هذه الدراسة بتقييم العديد من وسائط زراعة النفايات الزراعية لإنتاج الكتلة الحيوية لهذه البروبيوتيك، باستخدام الموز والبازلاء وقشور الأرز وقشور البطاطس. تم تحقيق أعلى إنتاج للكتلة الحيوية بواسطة (*Bacillus licheniformis* (CCASU-2024-65 عند ١,٩٧ جم / لتر مع وسط مستخلص قشر الموز، بينما سجلت *Bacillus subtilis* (CCASU-2024-6 إنتاجًا عاليًا للكتلة الحيوية بلغ ١,٥ جم / لتر مع وسط مستخلص قشر البطاطس. تم استخدام تصميم بلاكيت بورمان الإحصائي لفحص مكونات المغذيات وظروف الثقافة لهذه البروبيوتيك. كان الحد الأقصى لإنتاج الكتلة الحيوية لـ *Bacillus licheniformis* 2.7جم / لتر، تم تحقيقه في معامله رقم ٨، والتي كانت بها ظروف مثالية تتكون من درجة حموضة ٦، و ٥٠ جم / لتر من مستخلص قشر الموز، و ٥٠ جم / لتر من مستخلص قشر البازلاء، ونسبة تلقيح ١٠٪، ومدة تخمير ٤٨ ساعة لانتاج الكتلة الحيوية. أنتجت *Bacillus subtilis* ناتجًا ٣,٥٢ جم / لتر في المعامله رقم ١٢ والتي تتكون من ٥٠ جم / لتر من مستخلص قشر البطاطس و ٥٠ جم / لتر من مستخلص قشر البازلاء، ودرجة حموضة ٧، وحجم تلقيح ١٠٪، ومدة تخمير ٤٨ ساعة.