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Screening of effective factors in the growth of *Paenibacillus polymyxa* using Platelet-Berman experimental design

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ABSTRACT

Paenibacillus polymyxa is one of the microorganisms that has the ability to produce extracellular exopolysaccharides and antibiotics. Several factors, including culture medium content, carbon and nitrogen sources, pH, temperature, air velocity, and culture conditions, have an effect on the growth and production of higher cell mass, as well as the production of microbial metabolites. The purpose of this study was to investigate the growth rate of *P. polymyxa* in a culture medium containing molasses and to screen five components of the culture medium along with four factors of the fermentation conditions using the Plackett -Burman method to maximize cell mass production. The results showed that among the investigated variables, molasses brix, time, percentage of inoculation, amount of ammonium sulfate, stirring speed, and the amount of glucose and urea, as a first-order equatino, had a significant positive effect on bacterial growth and biomass production. Molasses brix medium was found to be more effective than other variables; however, pH and the amount of low-use elements had a negative effect on cell growth. The findings of this study indicated that molasses-based culture medium can be used as a cost-effective and suitable option for the growth of *P. polymyxa*.

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1- Introduction

Paenibacillus polymyxa is a Gram-positive bacterium belonging to the genus Paenibacillus. Members of this genus are facultative anaerobic, endospore-forming bacteria that were initially placed in the genus Bacillus and were later classified as a separate genus in 1993. *P. polymyxa*, which is naturally found in soil, is recognized for its ability to produce useful metabolic products such as antibiotics and polysaccharides [1]. Additionally, this organism has been considered a biocontrol agent and pest reducer in agriculture. *P. polymyxa* is capable of fixing nitrogen, improving soil quality, and acting as a plant growth stimulant. These properties underscore the importance of investigating the factors affecting the growth of this bacterium [2-4].

Various factors, including carbon sources, nitrogen, minerals, and environmental conditions such as temperature and pH, significantly influence bacterial growth. Diverse carbon sources such as sucrose, glucose, and starch are utilized for microbial growth, and the carbon to nitrogen (C:N) ratio in culture media is crucial for the production of high amounts of metabolites [5, 6]. Nitrogen is also recognized as one of the essential elements for the growth of microorganisms, with its effects being particularly evident during starvation and in enhancing production efficiency after the growth phase. Furthermore, certain minerals, including phosphorus, sulfur, and iron, serve as essential cofactors for enzymes and play fundamental roles in secondary metabolism. Temperature and pH are also significant environmental factors that greatly impact microbial growth and metabolite production [7, 8]. Given the high costs of metabolite production, there has been interest in using inexpensive substrates and by-products from the food and agricultural industries, such as molasses, as carbon sources. Molasses, a by-product of sugar factories, is employed as a raw material in medicine, industry, and agriculture [9, 10]. Sugar beet molasses is composed of a solution of sugar, organic, and inorganic substances in water, with a dry matter content of 77.74% (w/w). Total sugar, primarily sucrose, constitutes approximately 62.48%, ash 11.9%, and total nitrogen-containing compounds, mainly betaine and glutamic acid, 13.12%. The utilization of molasses in bacterial culture is particularly appealing due to its low cost and easy availability, especially in developing countries. It contains substantial amounts of sugar, protein, and minerals that can serve as energy and nutrient sources for bacteria. Additionally, the presence of amino acids, vitamins, and trace elements in molasses makes it a rich culture medium capable of facilitating bacterial growth [11, 12].

The Plackett-Burman method is an experimental technique that can be employed to screen and identify factors influencing the growth of microorganisms. Utilizing the Plackett-Burman method allows researchers to systematically investigate the factors that affect microbial growth, thereby gaining deeper insights into the biology of these organisms. This data can help researchers determine which conditions and factors have the greatest impact on bacterial growth, thereby enabling the optimization of culture conditions [13, 14].

Based on the above explanations, the main objective of this study is to investigate the effects of various factors (molasses, glucose, ammonium sulfate, urea, TES, pH, time, agitation, and inoculation rate) on the growth of *P. polymyxa* using the Plackett-Burman technique.

2- Materials and Methods

2.1. Microorganism Activation

To activate the microorganism, lyophilized *P. polymyxa* PTCC 1021, obtained from the Iranian Industrial Microorganism Collection Center, was transferred to a TSB culture medium under sterile conditions and incubated for 24 h at 30°C. The activated microorganism was then

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transferred to the surface of TSA culture medium and incubated under the same conditions (Fig. 1). The colonies obtained from this culture medium were utilized to carry out the fermentation process [15].

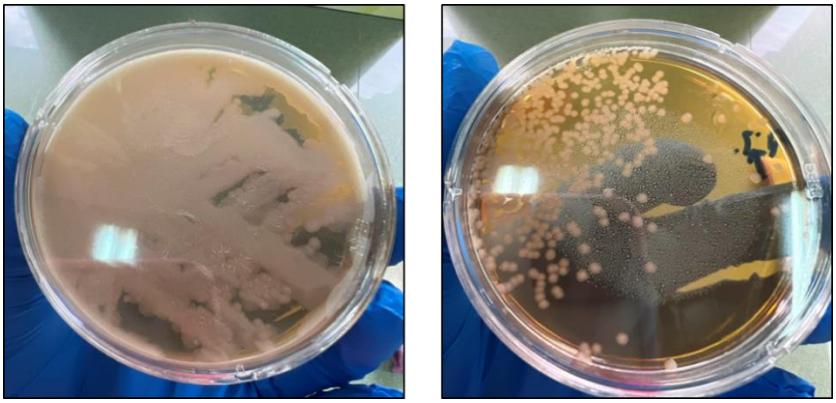

Fig. 1. *P. polymyxa* activated on the basic culture medium.

2.2. Bacterial Growth Curve

Following the initial activation of the microorganism in TSB, the microbial suspension was inoculated into the base culture medium and incubated at 30°C in a shaking incubator. The number of bacterial colonies was measured at various time intervals over six consecutive days, and a bacterial growth curve was plotted [16]. The basic fermentation medium consisted of 50 g/L glucose, 1 g/L yeast extract, 2 g/L monopotassium phosphate, 0.5 g/L dipotassium phosphate, 1 g/L ferric chloride hexahydrate, 1 g/L manganese chloride tetrahydrate, 1 g/L calcium chloride dihydrate, 0.5 g/L magnesium sulfate hexahydrate, and 1 g/L sodium chloride [16].

2.3. Screening of Factors Affecting Growth Using the Plackett-Burman Experimental Design

Given the significance of various growth factors on cell mass increase, a screening of factors affecting cell mass growth was performed based on five culture medium components: molasses (Brix), glucose (percent), ammonium sulfate (g/L), urea (g/L), and TES (which includes zinc chloride, manganese chloride tetrahydrate, nickel chloride hexahydrate, copper chloride tetrahydrate, cobalt chloride hexahydrate, and sodium molybdate dihydrate) (µg/L). Additionally, four fermentation condition parameters were evaluated: pH, time (h), agitation (rpm), and inoculation rate (percent). This screening was conducted based on the Plackett-Burman experimental design using statistical software (Design-Expert 7.0.0, Stat-Ease, Inc., Minneapolis, MN, USA). The selection of these variables was informed by a review of previous research and pretreatments, designed in a matrix format comprising 12 experiments [17].

2.4. Measurement of Cell Mass

At the conclusion of the fermentation period, the microbial culture medium was centrifuged at 8000×g for 20 min. Following centrifugation, the obtained cell mass was suspended in 2 M sodium hydroxide and placed on a shaker for one hour, after which it was centrifuged again. The pellet obtained in this stage was measured to determine cell mass concentration based on both wet and dry weight (g / L) [18].

2.5. Statistical Analysis

The initial screening for cell biomass production, based on four culture medium components and five fermentation condition parameters in shaker

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flasks, was performed using Design-Expert 7.0.0 software from Stat-Ease, Inc., Minneapolis, MN, USA, based on the Plackett-Burman test in a matrix format consisting of 12 experiments.

3- Results and Discussion

3.1. Analysis of the Growth Curve of *P. polymyxa*

Obtaining the microbial growth curve provides valuable information regarding the various phases of microbial growth, including the time of entry into the logarithmic phase, the duration of this phase, the time of entry into the stationary phase, the duration of the stationary phase, and the onset of the death phase. Therefore, based on the analysis of the microbial growth curve, the cultivation process can be appropriately timed for halting and product harvesting.

The growth of *P. polymyxa* in the basic culture medium was investigated, and the growth phases were plotted according to the results obtained. As illustrated in Fig. 2, during the first 12 and 24 h, the population of *P. polymyxa* reached approximately 3 and 7 logarithmic cycles, respectively. The characteristics of the inoculated microbial suspension and the bacteria's ability to metabolize carbon and nitrogen derivatives are critically important for the rapid exit from the lag phase. A short duration for the microorganism to reach the logarithmic phase and the maximum increase in growth are significant factors in the efficiency of bioproduction on an industrial scale, enhancing both the frequency of production processes and fermentation efficiency per unit volume [19]. The logarithmic phase continued until the third day, followed by the initiation of the stationary phase; in essence, the maximum growth in the basal medium was achieved at 72 h. The increase in *P. polymyxa* activity until the third day correlates with a decrease in available nutrients and micronutrient resources in the environment. Furthermore, on the third day, cell lysis begins with the secretion and activation of hydrolyzing enzymes; thus, alongside cell growth, cell death also occurs, leading to the commencement of the stationary phase. However, the impact of temperature and environmental factors such as pH can also influence the onset of the stationary phase, potentially reducing cell mass production [20]. According to the results from the growth curve of *P. polymyxa* in basal culture medium, the bacteria entered the death phase after 120 h (the fifth day).

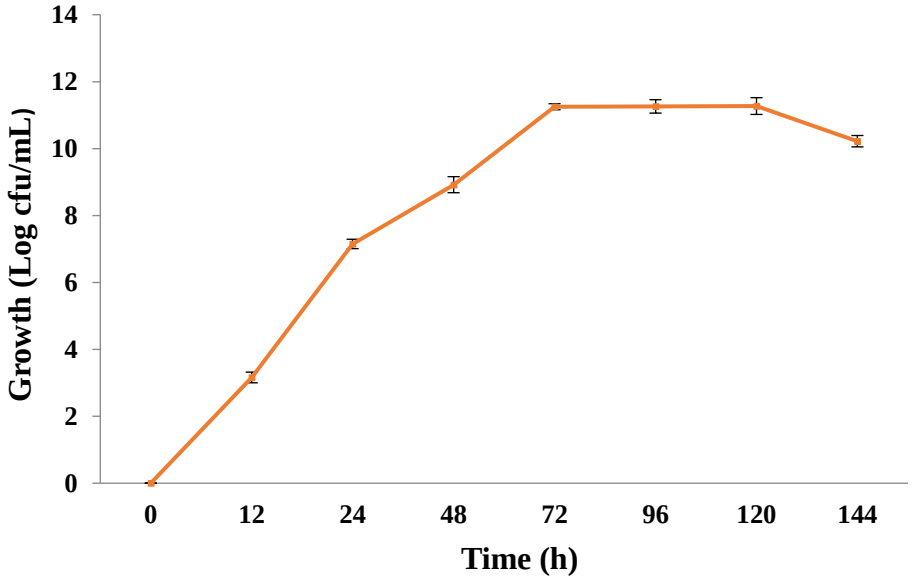

Fig. 2. Growth curve of *P. polymyxa* bacteria in basic culture medium.

3.2. Screening of Factors Affecting the Growth of *P. polymyxa*

In this study, a screening design based on the Plackett-Burman method was employed to

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identify variables affecting growth in molasses medium. This method is particularly advantageous when many potential factors (variables) exist, making it challenging to make decisions and select them for experimental design. By utilizing the minimum number of experiments, initial and valuable information about the system is obtained, which is beneficial for subsequent design stages [23].

The variables selected were based on prior research and pretreatments and were organized in a matrix format comprising 12 experiments. The independent variables included pH, rotation speed (rpm), fermentation time (h), the percentage of microorganism inoculation, molasses Brix, ammonium sulfate content (g/L), urea content (g/L), glucose content (%), and a solution of trace elements (µL/L). The Plackett-Burman matrix for evaluating bacterial cell mass production, along with the results of measuring the biomass produced, is presented in Table 1.

Table 1. Plackett–burman matrix to evaluate cell mass production

No.	TES (µL/L)	Time (h)	Glucose (%)	Inocolum (%)	Urea (g/L)	Ammonium sulfate (g/L)	Mollases (Bx)	Rotation (rpm)	pH	Wet Biomass (g/L)a	Dry Biomass (g/L)
	J	H	G	F	E	D	C	B	A		
1	1000	120	12	1	3	3	5	200	5	5.15	1.18
2	1000	120	2	10	1.5	1.5	5	200	5	6.13	1.19
3	100	72	2	1	1.5	1.5	5	100	5	5.56	1.42
4	1000	72	12	10	1.5	3	5	100	7	7.90	1.89
5	1000	120	2	10	3	1.5	25	100	7	17.78	4.35
6	100	72	12	10	3	1.5	25	200	5	14.03	3.68
7	1000	72	12	1	1.5	1.5	25	200	7	10.44	2.59
8	100	72	2	10	3	3	5	200	7	6.08	1.50
9	10000	72	2	1	3	3	25	100	5	12.08	3.08
10	100	120	12	1	3	1.5	5	100	7	7.12	1.73
11	100	120	2	1	1.5	3	25	200	7	11.45	2.75
12	100	120	12	10	1.5	3	25	100	5	12.46	3.19

Table 2 displays the analysis of variance (ANOVA) for the initial factors used in the screening. The model derived from the evaluation of independent factors on biomass production is significant, and with a coefficient of determination close to 0.9, the fitted model is deemed appropriate.

To assess the trend of changes in biomass production, it is crucial first to determine the appropriate model that fits the experimental data. Statistically, a model is suitable if the test of lack of fit is not significant and the test for fit is significant. Based on the p-values obtained for each factor in examining their effects on the amount of biomass production, it was determined that stirring speed, fermentation time, and inoculation percentage were significant at a 95%

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confidence level. Following these factors, molasses Brix, glucose percentage, and the amounts of urea and ammonium sulfate used were also important.

Shaker rotation refers to the impact of agitation speed and type in the fermentation process, aiding in the even distribution of nutrients and enhancing the growth conditions for microbial strains. The time allocated for fermentation can also influence biomass production; insufficient time may lead to inadequate or suboptimal production due to incomplete fermentation. Furthermore, the percentage of microorganisms introduced into the fermentation process or suspension will directly affect the amount of biomass produced. The appropriate inoculation percentage can enhance production.

According to the results obtained, the influence of Brix, which measures the concentration of dissolved sugars, indicates its direct correlation with the concentration of molasses and its effect on the fermentation process. Molasses serves as a significant source of sugar for microorganisms, and its optimal concentration, in conjunction with glucose as the primary sugar in the process, can augment biomass production. Additionally, nitrogen sources such as urea and ammonium sulfate can also impact biomass yield. To accurately investigate the effect of each factor, quantify it, and evaluate the response values, it is essential to analyze their variance table. The significance of the polynomial model equation was statistically evaluated through analysis of variance (ANOVA). ANOVA, along with the coefficient of variation, illustrates the effects of variables on determining the effective and ineffective parameters relevant to the response-related models.

Table 2. Variance analysis of variables influencing the growth of *P. polymyxa* based on the plackett–burman designs

Source	Sum of Squares	Mean Square	Mean Square	F value	P value	
Model	**84.19**	**9**	**8.42**	**0.716**	**0.0435**	**Significant**
pH (A)	**12.61**	**1**	**12.61**	**1.07**	**0.1488**	
Shaker (B)	**0.0075**	**1**	**0.0075**	**0.0006**	**0.0098**	
Mollases Bx (C)	**8.27**	**1**	**8.27**	**0.0703**	**0.0505**	
Ammonium sulfate (D)	**0.5689**	**1**	**0.5689**	**0.0484**	**0.0682**	
Urea (E)	**9.39**	**1**	**9.39**	**0.0798**	**0.0535**	
Inocolum (F)	**2.88**	**1**	**2.88**	**0.0244**	**0.0307**	
Glucose (G)	**9.68**	**1**	**9.68**	**0.0823**	**0.0530**	
Time (H)	**6.21**	**1**	**6.24**	**0.0531**	**0.0409**	
TES (J)	**2.72**	**1**	**2.72**	**0.0231**	**0.714**	
Residue	**11.76**	**1**	**11.76**			
Coefficient						**0.887**
Coefficient Variation						**14.52**

In the normal distribution curve of the Plackett-Burman design (Fig. 3), the importance of each factor under investigation is highlighted, with positive effects represented in orange and negative effects in blue. Among the variables studied, the Brix of the molasses used,

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fermentation time, inoculation percentage, ammonium sulfate content, stirring speed, glucose content, and urea content exhibited significant positive effects on bacterial growth and biomass production as demonstrated by a first-order equation. Notably, the effect of the molasses Brix was greater than that of the other variables; however, pH and the amount of trace elements used showed a negative impact on cell growth.

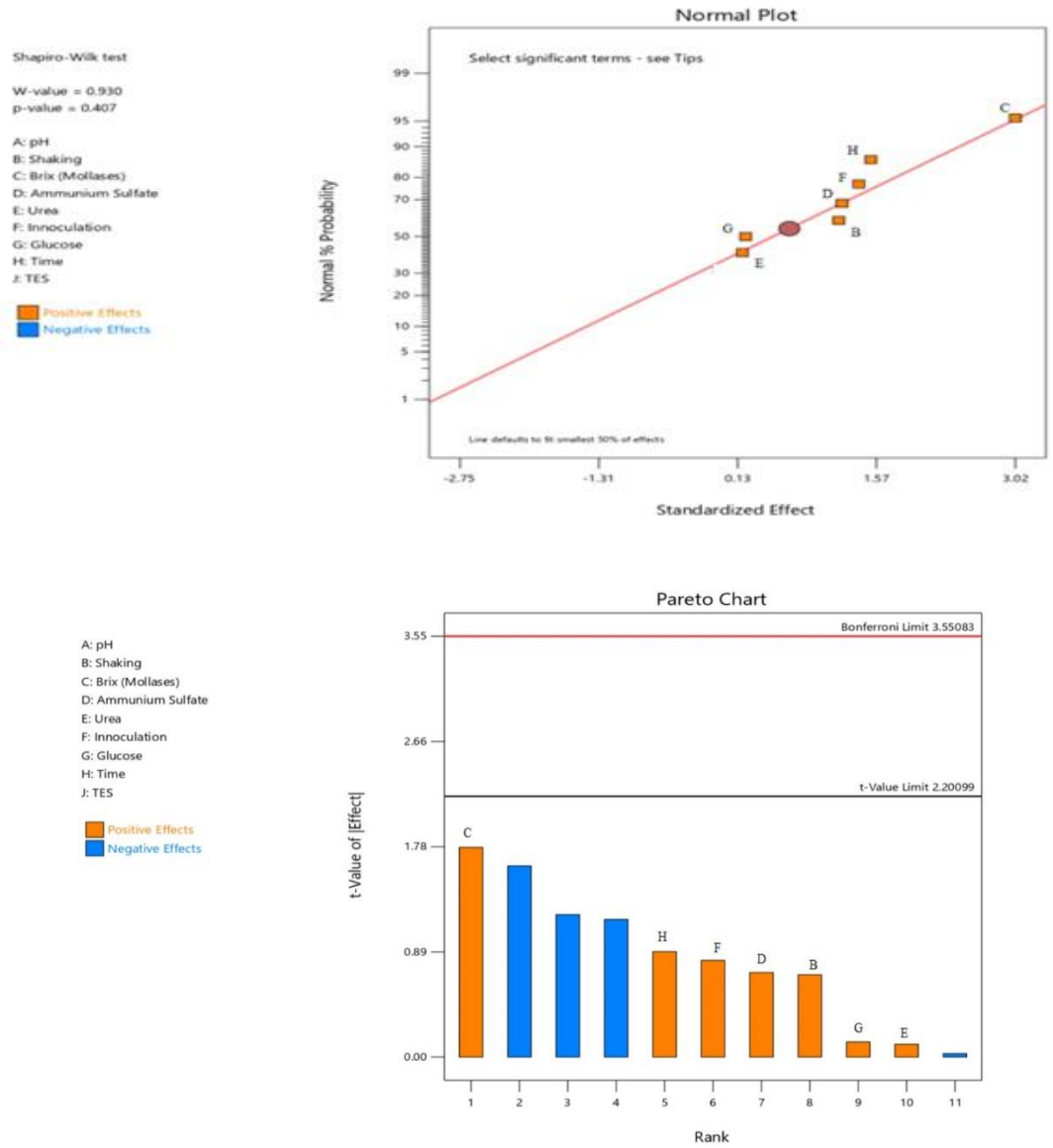

Fig. 3. The normal distribution curve of Burman's plackett plot related to the screening of independent variables influencing *P. polymyxa* biomass (Normal plot and Pareto chart).

Rafigh et al. (2014) investigated the impact of various parameters on cell mass production by the *P. polymyxa*, revealing that temperature and glucose concentration, as independent factors, had high coefficient values, indicating a significant linear effect on bacterial growth. The direct influence of six variables—temperature, pH, fermentation time, glucose, yeast extract, and stirring speed—on the efficiency of the response variable (biomass production) was clearly evident in their results. Specifically, they found that increasing the initial fermentation pH from 5.5 to 7.0 enhanced cell mass production; however, higher values (pH = 8.5) led to

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decreased production. Furthermore, their study revealed that extending the incubation time from 72 to 96 h increased biomass production, reaching a maximum at 96 h, beyond which longer durations did not positively affect biomass yield [16].

Additionally, the findings of Lee et al. (1999) indicated that the biomass production of Agrobacterium bacteria—similar to *P. polymyxa* in its capability to produce the exopolysaccharide cordlan—increased significantly with stirring speeds rising from 120 to 150 × rpm. The highest biomass production was recorded at a stirring speed of 150 × rpm; however, at 120 × rpm, biomass production was lower due to limited oxygen transport. Conversely, biomass production was reported to be reduced at 180 × rpm, likely due to bacterial degradation caused by excessive shear forces [24].

The Plackett-Burman design has been effectively employed in various studies to optimize the culture medium of microorganisms within the Bacillus family, which are closely related to Paenibacillus. For example, Shi et al. (2024) focused on optimizing the fermentation medium and growth conditions of *Bacillus velezensis* BHZ-29, reporting significant enhancements in both biomass production and antibacterial potency for this strain. Their experimentation using the Plackett-Burman design identified crucial factors impacting bacterial viability and antibacterial effectiveness, while the Box-Behnken design was utilized to refine optimal growth conditions for *B. velezensis* BHZ-29. The findings indicated that molasses, peptone, and magnesium sulfate were pivotal factors significantly influencing bacterial growth and antibacterial activity. Specifically, the number of viable cells increased from 7.83×10^9 CFU/mL to 2.17×10^{10} CFU/mL, and the antibacterial titer improved from 111.67 to 153.13 mm/mL. Post-optimization, the fermentation conditions for *B. velezensis* BHZ-29 were established as follows: temperature 25.57 °C, pH = 7.23, cultivation time 95 h, rotation speed 160 × rpm, inoculum 2%, and fermentation volume 100 mL. Following these optimizations, the number of viable bacteria rose to 3.9×10^{10} CFU/mL, and the bacteriostatic titer increased to 158.85 mm/mL [25].

A study by Ghasemi and Ahmadzadeh (2013) investigated methods for screening and optimizing fermentation processes to simultaneously enhance biomass production and the efficacy of a biocontrol metabolite derived from *Bacillus subtilis* UTB96. Utilizing a Plackett-Burman design, the researchers effectively examined various environmental factors influencing cell mass production, focusing on pH, temperature, and the carbon-to-nitrogen (C/N) ratio as key parameters. The determined optimal conditions—pH of 7, temperature of 30°C, and a C/N ratio of 23:1—align well with growth profiles commonly observed in many Bacillus species. Maintaining a balanced pH is particularly crucial for microbial metabolism, as it facilitates enzymatic activity and nutrient uptake. The selected C/N ratio likely creates a balanced environment that aids in the synthesis of essential molecules, thereby contributing to cell mass accumulation. A prominent finding of this study was the significant increase in cell mass when bacteria were cultured under these optimal conditions. The researchers noted that applying screening factors affecting cell mass growth, along with optimizing culture conditions in both semi-industrial bioreactors and laboratory settings, positively influenced the reduction of the lag phase of bacterial growth. This observation implies that providing an optimal environment can accelerate bacterial adaptation to new conditions, subsequently increasing productivity. Reducing the lag phase is especially advantageous in biotechnological applications, as time efficiency significantly impacts economic profitability [26].

Praharyawan et al. (2014) employed the Plackett-Burman design to screen environmental compounds aimed at enhancing biosurfactant production by Bacillus sp. DSW17. This methodology effectively identified key factors

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influencing biosurfactant yield and represents a critical step in optimizing fermentation processes for biosurfactant production. Out of the 11 compounds investigated, 9 were found to significantly affect biosurfactant production. Notably, $FeSO_4 \cdot 7H_2O$, $NaNO_3$, $CaCl_2 \cdot 2H_2O$, K_2HPO_4, and sucrose demonstrated a direct correlation with biosurfactant production. Nitrogen sources, such as $NaNO_3$, are essential for synthesizing proteins and nucleic acids, whereas carbon sources like sucrose provide the energy necessary for bacterial growth and biosurfactant synthesis. Conversely, $ZnSO_4 \cdot 7H_2O$, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ showed an inverse relationship with biosurfactant production, suggesting that high concentrations of these compounds might inhibit biosurfactant synthesis within the studied experimental range [27].

Rajendran et al. (2007) highlighted the effectiveness of the Plackett-Burman design in statistically evaluating culture medium components to enhance cell mass and lipase production by *Bacillus sphaericus*. By analyzing twelve environmental components in a submerged fermentation process, this study effectively identified key factors significantly affecting lipase production. Among the components evaluated, glucose, olive oil, peptone, NaCl, and $MnSO_4 \cdot H_2O$ emerged as the most important for lipase activity. The role of glucose and olive oil as carbon sources proved particularly significant; glucose serves as a primary energy source, enhancing cellular respiration and biomass growth, while olive oil supplies essential fatty acids crucial for lipase production. Peptone's role as a nitrogen source is also vital, providing amino acids necessary for protein synthesis, including lipase. Achieving a maximum lipase activity of 2.82 U/mL in the fermentation process underscores the potential of *B. sphaericus* as an effective lipase producer [28].

4-Conclusion

P. polymyxa is a naturally occurring and beneficial Gram-positive bacterium found in soil, known for its capability to produce a variety of antibiotics and polysaccharides, including curdlan. Consequently, providing a solution for the enhanced growth of this strain in low-cost culture media could culminate in the production of a final product at reduced costs. This study focuses on investigating the effects of various carbon sources, nitrogen, and environmental conditions, placing particular emphasis on utilizing molasses as an inexpensive and accessible carbon source to promote the growth of this bacterium. The results from the screening tests demonstrated that stirring speed, fermentation time, and inoculation percentage significantly influenced the rate of cell mass production ($P<0.95$) more than other parameters examined. Further research is warranted to devise a suitable strategy for promoting the growth of *P. polymyxa* in a culture medium containing molasses, aimed at achieving higher cell mass production and, consequently, the efficient generation of desired metabolic products.

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غربالگری فاکتورهای موثر در رشد باکتری *Paenibacillus polymyxa* با استفاده از طرح آزمایشی پلاکت-برمن

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چکیده

باکتری *Paenibacillus polymyxa* از جمله میکروارگانیسم‌های است که توانایی تولید اگزوپلی‌ساکاریدهای خارج سلولی و نیز آنتی‌بیوتیک‌ها را دارا می‌باشد. عوامل متعددی از جمله محتوای محیط کشت، منبع کربن و نیتروژن، pH، دما، سرعت هوا و شرایط کشت بر رشد و تولید توده سلولی بالاتر و نیز تولید متابولیت‌های میکروبی تأثیر دارند. هدف از این مطالعه بررسی میزان رشد باکتری *P. polymyxa* در محیط کشت حاوی ملاس و همچنین غربالگری ۵ جزء محیط کشت و ٤ فاکتور شرایط تخمیر با استفاده از روش پلاکت-برمن جهت به حداکثر رساندن میزان تولید توده سلولی بود. نتایج نشان داد از بین متغیرهای مورد بررسی بریکس ملاس مورد استفاده، زمان، درصد تلقیح، میزان سولفات آمونیوم، سرعت هم‌زدن، میزان گلوکز و اوره به‌صورت معادله درجه اول اثر مثبت معنی‌داری بر رشد باکتری و تولید زیست‌توده دارد که از این میان اثر بریکس ملاس مورد استفاده بیش از سایر متغیرها است؛ این درحالی است که pH و میزان عناصر کم مصرف مورد استفاده اثر منفی بر رشد سلولی داشتند. نتایج این مطالعه نشان داد محیط کشت برپایه ملاس می‌تواند به عنوان یک گزینه ارزان قیمت و مناسب برای رشد باکتری *P. polymyxa* مورد استفاده قرار گیرد.

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