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IDENTIFICACIÓN DE LAS VARIABLES FISICOQUÍMICAS QUE INFLUYEN EN LA PRODUCCIÓN DE PHB POR *Bacillus megaterium* MNSH1-9K-1

IDENTIFICATION OF THE PHYSICOCHEMICAL VARIABLES INVOLVED IN THE PRODUCTION OF PHB BY *Bacillus megaterium* MNSH1-9K-1

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Resumen

El presente estudio se enfocó en la identificación de las variables fisicoquímicas que influyen en la producción de polihidroxibutirato (PHB) por la cepa *Bacillus megaterium* MNSH1-9K-1, utilizando un medio de cultivo elaborado a partir de desechos agroindustriales, específicamente residuos de cáscara de naranja. Estudios previos demostraron que la cepa mencionada tiene la capacidad de producir este biopolímero (BP) en medios de bajo costo, lo cual pudiera ser un componente importante para disminuir los costos de producción. El presente estudio evaluó el efecto de algunos factores fisicoquímicos de crecimiento que han sido reportados anteriormente como relevantes para mejorar la producción bacteriana de PHB, utilizando para ello un diseño estadístico de Plackett-Burman; además, los datos fueron analizados a través de un modelo de regresión de superficie de respuesta. Los resultados obtenidos serán de utilidad para buscar la optimización de este proceso biotecnológico, con el enfoque de establecer una alternativa viable para la sustitución del uso de BP convencionales y, de esta manera, promover la disminución del impacto ambiental ocasionado por los residuos plásticos.

Palabras clave: Biopolímeros, PHB, *Bacillus megaterium*, medios de cultivo de bajo costo, residuos de cáscara de naranja.

Abstract

The present study focused on the identification of the physicochemical variables that influence the production of polyhydroxybutyrate (PHB) by *Bacillus megaterium* strain MNSH1-9K-1, using a culture medium elaborated from agro-industrial waste, specifically orange-peel residues. Previous studies demonstrated that the strain can produce this biopolymer (BP) in low-cost media, which could be an important component to reduce production costs. The present study evaluated the effect of some physicochemical growth factors that have been previously reported as relevant to improve the bacterial production of PHB, using a Plackett-Burman statistical design; in addition, the data was analyzed through a response surface regression model. The results obtained will be useful to seek the optimization of this biotechnological process, with the focus of establishing a viable alternative to replace the use of conventional BP and, in this way, promote the reduction of the environmental impact caused by plastic waste.

Keywords: Hydrogels, Polyacrylamide, Polymers

Introduction

The production of plastics and the use of synthetic polymers is still growing exponentially nowadays. Due to their low degradability, most discarded plastics persist in the environment, negatively impacting ecosystems and threatening biodiversity and habitats **(Buteler, 2020)**.

Because of this severe problem, there is a continuous search for feasible alternatives to diminish the impact produced by conventional plastics, like the generation of biopolymers (BP). Polyhydroxyalkanoates (PHA), and specifically polyhydroxybutyrates (PHB), present similar characteristics to polypropylene, with the advantages of biodegradability and barrier properties (water insolubility and low permeability to O_2, H_2, and CO_2) **(Leja & Lewandowicz, 2010)**.

Among the wide variety of bacteria capable of producing PHA, it has been previously reported that *Bacillus megaterium* strain MNSH1-9K-1 presents relevant characteristics for biotechnological purposes **(Rivas-Castillo et al., 2017, 2018, 2019a)**; more precisely, strain MNSH1-9K-1 possesses an enhanced capability for PHB production compared to other bacterial producers reported to date **(Angeles-Padilla, 2019; Rivas-Castillo et al., 2019b, 2022)**. In addition, it has been observed that this high BP production can be sustained in a low-cost culture medium produced from orange peel residues **(Angeles-Padilla, 2019; Figueroa-Ocampo, 2019)**.

Thus, the present study aimed to identify the main physicochemical variables that may influence PHB production by *B. megaterium* MNSH1-9K-1 using a Plackett-Burman experimental design, with the objective of potentiating this biotechnological production process.

Experimental section

Microorganism and growth conditions

B. megaterium MNSH1-9K-1 was isolated from a mining site in Guanajuato, Mexico **(Rivas-Castillo et al., 2019a)**. Pre-inoculums were grown for 12-24 h in 125-mL flasks containing 50 mL of Nutrient Broth (NB), at 30°C and 120 rpm. Afterward, total cell counts were performed using a Neubauer chamber to inoculate experimental sets with $1x10^6$ total cells, which were grown for 48 h **(Gouda et al., 2001; Angeles-Padilla, 2019)**. The control condition (C) was evaluated in 50 mL of NB at 30°C and 150 rpm **(Angeles-Padilla, 2019)**; for other growth conditions, factors were varied according to the experimental design proposed (Table 1).

Preparation of culture medium from orange-peel residues

Oranges were bought from a supermarket. Peels were removed from the fruits and the culture medium was prepared following the protocol previously described **(Rivas-Castillo et al., 2019b)**, refrigerating it until further use. Subsequently, the medium was supplemented as specified for the different conditions assessed and sterilized by tyndallization to avoid the caramelization of the included components **(Brown, 1979; Fürch et al., 2007)**. In the case of the soluble starch, dry sterilization was applied to prevent its gelatinization, performing it in a drying oven for 20 min at 200°C **(Nagy et al., 2021)**; letting it cool at environmental temperature, and adding it to the culture medium after sterilization (**Pérez-Uz et al., 2010)**.

PHB extraction

The sodium hypochlorite-chloroform method was used **(Rivas-Castillo et al., 2019b; Valdez-Calderón et al., 2022)**, as follows: The samples were centrifuged at 4000 rpm for 20 min to obtain the biomass. Then, the liquid phase was discarded, and sodium hypochlorite and chloroform were added to the pellet in a 1:10 (w/v) proportion each, mixing thoroughly until the bacterial cells were completely resuspended. The samples were incubated at 30°C and 120 rpm for 90 min, and the upper phase was removed using a Pasteur pipette. To precipitate the BP, cold methanol was added to the lower phase in a 1:10 proportion (w/v), mixed, and let stand for 24 h at 4°C. Afterward, the samples were heated in a water bath at 65°C until the liquid was reduced to 5-10%, and subsequently transferred to dry heat (oven at 65°C) until dryness. Once completely dried, the samples were cooled at environmental temperature and the PHB obtention was determined gravimetrically.

Experimental design

The physicochemical variables to assess were determined based on the factors previously reported to have a significant impact on PHA production (**Omar et al., 2001; Yanti et al., 2009; Johnson et al., 2010; Younis et al., 2010; Shah., 2014; Peña-Jurado et**

al., 2019; Soruba et al., 2019; Suryawanshi et al., 2020; El-Kadi et al., 2021; Khatami et al., 2021; Drusilla et al., 2022; Samal et al., 2023). A complete Plackett-Burman factorial design was used, to evaluate the effect of (Table 1): pH, rpm, temperature, inoculum (total cells), the volume of the medium in the flask, ammonium sulfate, cane molasses, casein peptone, ethanol, fructose, glucose, glycerol, lactic casein, sodium acetate, sodium nitrate, soluble starch, sucrose, and whey in two different conditions (B, untreated; and B_1, treated by centrifugation at 3000 rpm for 20 min to remove solids. In the case of the growth factors, a lower and an upper limit were established; for nutritional variables, presence/absence conditions were used (n=2).

Statistical analyses

The basic statistical analyses were performed using the commercial software Origin Pro 9.0, and the lower-case letters indicate groups of data that are significantly different ($P \leq 0.05$). To evaluate the influence of physicochemical variables in PHB production, Minitab® 19 software was used to both elaborate and analyze the Plackett-Burman experimental design.

Results and Discussion

Production of PHB under different growth conditions.

As can be observed in Figure 1, from the 44 different growth conditions evaluated (Table 1), the BP production changed differentially depending on the growth factors and the type and quantity of carbon and nitrogen sources used to enrich the medium. The highest PHB production yields were obtained from conditions 30 (112.62 g/L); followed by condition 21 (91.14 g/L), and condition 8 (68.25 g/L). Thus, up to a 9-fold increment in PHB production was achieved in condition 30, whose growth conditions were: pH 6, 30°C, 150 mL, 150 rpm, and an inoculum of $1x10^8$ total cells; and supplemented with (g/L): soluble starch, 100; sucrose, 100; cane molasses, 100; sodium nitrate, 10; lactic casein, 10; sodium acetate, 30; untreated whey (B), 150; and, treated whey (B_1), 150. These results could be attributed to the cellular stress produced by the specific growth conditions which, in some cases like in condition 30, favored PHB production, such as anoxic conditions, the saturation of carbon content, and the lack of nitrogen. It is important to mention that the carbon:nitrogen relationship found in condition 30 is 8:1.

Statistical analysis to determine significant factors for PHB production.

Results were analyzed by a response surface regression model in order to determine which variables have an impact on the production process (Table 2). For this purpose, all the variables were considered as continuous, and factors with $P \leq 0.05$ were determined as significant. In addition, P positive and negative values were examined.

The second column (Effect) in Table 2 specifies the influence of each variable on the PHB production process, being the soluble starch and the sodium nitrate the factors with the highest effect, both presenting a positive influence on the process.

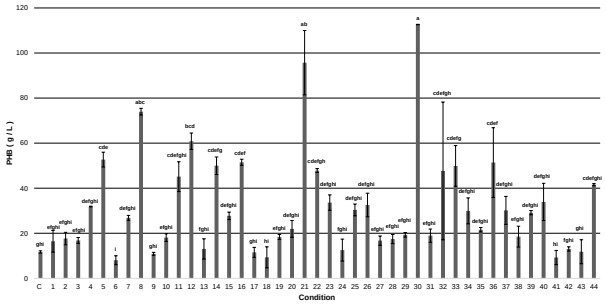

Figure 1. PHB production under the diverse growth conditions evaluated. C, control.

Additionally, to these two factors with high positive values, there were observed other variables with positive effects, like rpm, volume of medium, glucose, sucrose, cane molasses , casein peptone, yeast extract, ammonium sulfate, glycerol, sodium acetate, treated whey (B_1), and the number of total cells in the inoculum ; all these variables show a favorable impact on production yield, however, they cannot be considered as significant due to their P value (> 0.05).

There are also factors showing negative values on the effect, which can be classified as detrimental for the response sought; that means that their presence, in high values or concentrations, affects PHB production, like pH, temperature, fructose, ammonium nitrate, ethanol, and untreated whey (B).

Like P, T values help to determine the significance of the variables, the higher the T value, the higher the significance. Complementing P interpretation (≤ 0.05), the highest T-significant values are observed in soluble starch and sodium nitrate. The third column in Table 2 (Coef) describes the degree and direction of

the relationship between each term evaluated and the response variable, considering the mean value of the effect. Then, the factors with positive Coef values may have a significant direct effect on PHB production, while terms with negative values affect the response.

Table 2. Codified coefficients.

Term	Effect	Coef[a]	Coef EE[b]	*T* value	*P* value	VIF[c]
Constant		31.66	2.73	11.59	0.000	
pH	-5.91	-2.96	2.83	-1.04	0.308	1.08
Temperature	-9.51	-4.75	2.93	-1.62	0.119	1.15
Glucose	1.95	0.98	2.86	0.34	0.736	1.10
Soluble starch	22.42	11.21	2.92	3.84	0.001	1.14
Sucrose	8.95	4.48	2.85	1.57	0.130	1.09
Fructose	-6.41	-3.20	2.82	-1.14	0.267	1.06
Cane molasses	1.28	0.64	2.94	0.22	0.830	1.16
Casein peptone	2.16	1.08	2.88	0.37	0.712	1.11
Ammonium nitrate	-9.16	-4.58	2.94	-1.56	0.133	1.16
Yeast extract	3.23	1.62	2.99	0.54	0.595	1.20
Sodium nitrate	12.07	6.03	2.88	2.09	0.048	1.11
Lactic casein	5.61	2.80	2.81	1.00	0.330	1.06
Ammonium sulfate	3.18	1.59	2.87	0.55	0.586	1.10
Glycerol	0.16	0.08	2.92	0.03	0.979	1.14
Absolute ethanol	-4.21	-2.10	2.86	-0.73	0.471	1.10
Sodium acetate	1.83	0.92	2.87	0.32	0.752	1.10
Whey B	-10.77	-5.39	2.83	-1.90	0.071	1.08
Whey B_1	2.78	1.39	2.82	0.49	0.626	1.06
Total cells	4.22	2.11	2.86	0.74	0.468	1.10
Volume of the medium	10.31	5.16	2.88	1.79	0.087	1.11
RPM	11.60	5.80	2.86	2.03	0.054	1.09

[a]Coef, Coefficient

[b]Coef EE, Standard error of the coefficient

[c]VIF, Variance Influence Factor

In the case of the subsequent column, corresponding to the standard error of the Coef value (Coef EE), it shows the variability of the Coef values to evaluate if the effect of each factor is reliable. As can be observed, Coef EE is similar for all the variables assessed. Finally, the Variance Influence Factor (FIV) allows the identification of multicollinearity; that is, the correlation between the variables included in the analysis. Because all FIV are around 1, and then all are < 5, there is no correlation between the factors, establishing that the effects obtained are statistically significant.

Then, to graphically observe the effect that the variables have on PHB production, the standardized effects were assessed (Figure 2), for which the diagonal line in the middle indicates the separation between positive and negative values; the values on the left side have a negative impact on the process, whereas the ones on the right represent a positive effect. Even more, the factors that can be considered highly significant are the ones that appear farther from the 0 (cero) value on the X-axis and are presented in red: D, soluble starch, and L, sodium nitrate.

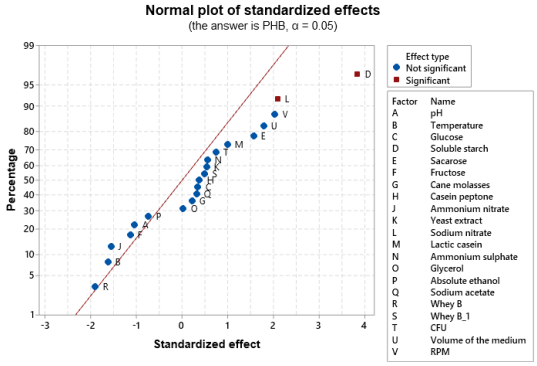

Figure 2. The normal plot of standardized effects.

Finally, Figure 3 corresponds to the Pareto diagram of the data, to visualize the most significant factors for the PHB production process in terms of the *P* value, showing influencing variables regardless of whether they are beneficial or counterproductive for the response analyzed. Again, it can be observed the high impact of starch and sodium nitrate for PHB production.

The growth conditions used in condition 30 exert the most suitable scenario for PHB production through the study, considering that BP are generated under stressful circumstances of bacterial growth. The analysis of the experimental design showed that soluble starch and sodium nitrate are the two variables with the highest influence on the production, reflecting that the microorganism prefers a complex carbohydrate source to simple ones to enhance the production process.

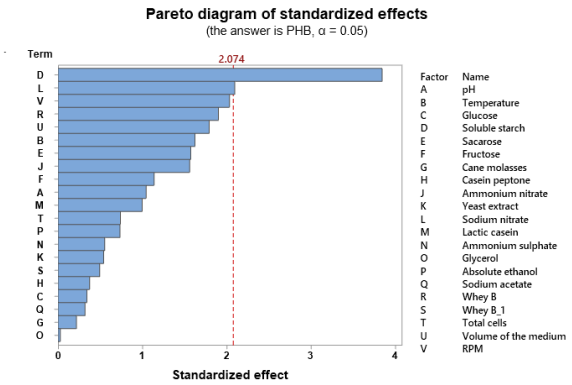

Figure 3. Pareto diagram of the variables assessed.

Although starch preference by MNSH1-9K-1 under the conditions assessed is uncertain, the use of renewable carbon sources for PHB biosynthesis may support the diminishing of production costs, and bacterial species like *Escherichia coli* and *Vibrio alginolyticus* have been engineered to use starch as carbon source for BP production (Bhatia et al., 2015; Li et al., 2023); *B. megaterium* MNSH1-9K-1 has this intrinsic capability. Regarding sodium nitrate, there has been previously reported that the presence of this compound may enhance the metabolic pathway of PHB synthesis by increasing pyruvate production, as was observed in *Halomonas* sp. strain KM-1(Kawata et al., 2016).

Additionally, it seems possible that the diminishing of available oxygen favored the production of BP (caused by a decrease in the available air in the free space of the flask and the aeration by orbital agitation), as observed for other bacterial species (Saharan et al., 2014; Madhusoodanan et al., 2022). However, considering that the experimental design used in the study is based on two levels (maximum and minimum), it will be strongly recommended to further evaluate some factors (in addition to soluble starch and sodium nitrate) with different central points to corroborate their influence, like in the case of those whose *P* values are close to the significance limit: rpm, temperature, volume of the medium, ammonium nitrate, sucrose, and untreated whey (B); considering that these latter factors have been identified as significant for other PHA production processes.

Conclusions

There was achieved a 9-fold increase in the PHB production by *B. megaterium* MNSH1-9K-1, observing that the three growth conditions (30, 28, and 32) with the highest production yields have similar characteristics, as pH 6 and the addition of soluble starch and sodium nitrate. In addition, condition 30 seems to possess other key attributes, like lower aeration and lower temperature.

Overall, the obtained results set the precedents in the path for the optimization of the process, which will encompass microbiological, molecular, and statistical approaches. The response surface regression analysis performed may serve as a guide for the gradual variation of the factors that affect the production process.

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Table 1. Different growth conditions evaluated.

No	pH	Temp (°C)	Glucose (g/L)	Starch (g/L)	Sucrose (g/L)	Fructose (g/L)	Cane molasses (g/L)	Casein peptone (g/L)	Ammonium nitrate (g/L)	Yeast extract (g/L)	Sodium nitrate (g/L)	Lactic casein (g/L)	Ammonium sulfate (g/L)	Glycerol (g/L)	Ethanol (g/L)	Sodium acetate (g/L)	Whey B (g/L)	Whey B_1 (g/L)	Inoculum (Total cells))	Medium in flask (mL)	Agitation (rpm)
1	8	40	0	0	10	0	0	0	0	1	1	0	1	0	3	3	15	0	$1x10^8$	50	150
2	6	40	0	0	0	0	10	0	1	1	1	1	0	3	0	0	0	0	$1x10^8$	50	150
3	6	30	10	0	0	10	10	1	0	1	1	0	0	0	3	0	15	15	$1x10^8$	50	25
4	8	30	0	0	0	10	10	0	1	0	0	0	1	0	0	3	15	15	$1x10^3$	50	150
5	8	30	0	10	0	0	10	1	0	1	0	0	1	3	0	0	15	15	$1x10^8$	150	150
6	6	40	0	0	0	10	0	0	0	0	1	1	1	3	3	0	15	15	$1x10^3$	150	25
7	8	40	0	10	10	0	10	1	0	0	0	1	1	0	3	0	0	15	$1x10^3$	50	25
8	6	40	10	10	0	10	0	0	0	1	0	1	1	0	0	3	0	15	$1x10^8$	50	150
9	6	40	0	0	10	0	0	1	0	1	0	0	0	3	0	3	15	15	$1x10^3$	50	25
10	8	30	0	0	0	0	0	0	1	1	0	1	0	0	3	3	0	15	$1x10^8$	150	25
11	6	30	0	10	10	10	10	0	1	1	1	0	1	3	3	3	0	15	$1x10^8$	50	25
12	8	30	10	10	0	10	10	0	1	0	1	1	1	0	3	0	0	0	$1x10^8$	150	150
13	8	40	10	0	0	0	0	1	0	0	0	1	0	0	0	0	15	0	$1x10^3$	150	150
14	8	30	10	10	0	0	10	0	0	1	1	1	0	3	3	3	15	15	$1x10^3$	50	150
15	8	30	0	0	10	0	10	1	1	0	1	0	0	3	3	0	15	0	$1x10^3$	150	25
16	6	40	10	10	10	10	10	1	0	0	1	0	1	3	0	0	15	0	$1x10^3$	50	150
17	6	30	10	0	10	0	10	0	1	1	0	1	1	0	0	0	15	0	$1x10^3$	150	25
18	6	40	0	10	10	10	0	0	1	1	0	0	0	0	3	0	15	15	$1x10^3$	150	150
19	6	30	10	0	0	10	0	0	1	1	1	0	0	3	0	3	15	0	$1x10^8$	150	150
20	8	40	10	0	10	10	10	1	1	1	1	1	1	3	0	3	15	15	$1x10^8$	150	25
21	6	40	10	10	10	0	0	1	0	1	1	1	0	3	3	0	0	0	$1x10^3$	150	25
22	8	40	0	10	10	0	0	0	1	0	0	1	1	3	0	3	0	0	$1x10^3$	150	150
23	6	30	0	10	0	0	0	0	0	0	0	0	0	0	3	0	0	0	$1x10^3$	50	150
24	6	40	0	0	10	10	0	1	1	0	0	0	1	0	0	0	0	0	$1x10^8$	150	25
25	8	40	0	10	0	10	10	0	0	1	1	0	0	0	0	3	0	0	$1x10^3$	150	25
26	8	40	0	10	0	10	0	1	0	0	1	0	0	3	3	3	0	15	$1x10^8$	50	150
27	8	40	10	0	0	10	0	1	1	1	0	0	1	3	3	0	0	0	$1x10^3$	50	25
28	8	30	0	10	10	10	0	0	0	1	0	1	0	3	0	0	15	0	$1x10^8$	50	25
29	8	40	10	10	0	0	0	0	1	0	1	0	0	0	0	0	15	15	$1x10^8$	50	25
30	6	30	0	10	10	0	10	0	0	0	1	1	0	0	0	3	15	15	$1x10^8$	150	150
31	6	30	10	0	10	0	0	1	1	0	0	1	1	3	3	0	15	15	$1x10^8$	50	150
32	6	30	0	10	0	0	0	1	1	1	1	1	1	0	0	3	15	0	$1x10^3$	50	25
33	8	30	10	10	10	0	10	1	1	1	0	0	0	0	0	3	0	0	$1x10^3$	50	150
34	8	30	0	0	10	10	0	1	1	1	1	1	0	0	0	0	0	15	$1x10^3$	50	150
35	8	30	10	10	10	10	0	1	0	0	0	0	1	0	3	3	15	15	$1x10^3$	150	25
36	6	30	0	0	10	10	10	1	0	1	0	1	1	3	3	3	0	0	$1x10^3$	150	150
37	6	40	10	10	0	0	10	0	0	0	0	0	1	3	3	3	15	0	$1x10^8$	150	25
38	6	40	10	0	0	0	10	1	1	0	1	0	1	0	3	3	0	15	$1x10^3$	150	150
39	8	40	10	0	10	10	10	0	0	0	0	0	0	3	3	0	0	15	$1x10^8$	150	150
40	6	30	10	10	0	10	10	1	1	0	0	1	0	3	0	0	0	15	$1x10^3$	150	25
41	6	40	0	10	0	10	10	1	1	0	0	1	0	0	3	3	15	0	$1x10^8$	50	150
42	8	20	10	0	0	0	10	1	0	0	1	1	1	3	0	3	0	0	$1x10^8$	50	25
43	6	40	10	0	10	10	0	0	1	0	1	1	0	0	3	3	0	0	$1x10^3$	50	25
44	8	30	10	0	10	0	0	0	0	1	1	0	1	3	0	0	0	15	$1x10^3$	150	25