

Improvement of the PHB Production Process by *Bacillus Megaterium* MNSH1-9K-1 in a Medium Prepared from Orange-peel Residues

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Polyhydroxybutyrate (PHB) is a thermoplastic biopolymer synthesized by a wide range of microorganisms, such as *Bacillus cereus* ARY73, *Klebsiella pneumoniae* E22, and *Pseudomonas putida*. Although PHB is a feasible option to substitute conventional plastics for specific purposes, this substitution has not been satisfactory due to its high production cost mainly due to the materials used for bacterial growth and the low yields obtained. *Bacillus* members are known for their ability to produce PHB, as they can accumulate this biopolymer in up to 80% of their weight, specifically under stressful growth conditions. *B. megaterium* strain MNSH1-9K-1 can produce up to twice as much PHB compared to other high-producing strains reported to date in a low-cost medium prepared from orange peels, and it was previously shown that this production can be enhanced by modifying some growth conditions using a presence/absence experimental design. Then, to identify the quantities of specific factors that can improve this PHB production by MNSH1-9K-1, different experimental scenarios were evaluated in the present work using the low-cost medium, including carbon and nitrogen concentrations, temperature, and aeration. PHB production was improved up to three times with a C/N ratio of 16:1. In addition, the significance of the evaluated variables for PHB production was determined by a response surface regression model. Due to the high production yield obtained with the use of a low-cost medium, the PHB production process using *B. megaterium* MNSH1-9K-1 may be a promising alternative to further explore its scale-up potential.

1. Introduction

Polymers are used for a wide range of industrial and commercial applications, such as agriculture, medicine, textiles, and construction. However, the negative effects they have on the environment due to their slow degradation are broadly known, causing soil, water, and air pollution. Thus, it is necessary to replace conventional polymers with more environmentally friendly materials, such as biopolymers (BP), which degrade in short periods when exposed to biologically active environments (Martínez-Herrera et al., 2023). PHB is a BP considered a feasible alternative for substituting conventional plastics, being polyesters synthesized by various organisms, including prokaryotes. PHB can be produced from renewable sources; they were recognized in 1920 by Trabuchet-Lemoigne as intracellular granular components accumulated in the cytoplasm under certain growth conditions, specifically under stressful ones, and are synthesized through fermentation processes of sugars or lipids. Some bacterial genera that can produce PHB are *Pseudomonas*, *Azotobacter*, and *Bacillus* (Mohanrasu et al. 2020).

Among polyhydroxyalkanoates (PHA), PHB is a viable option to replace conventional polymers for different purposes due to its characteristics, such as thermoplasticity and malleability (Martínez-Herrera et al., 2023). However, production costs represent a problem; this cost, which is approximately 6 to 15 dollars/Kg, can be ten

times higher than that of conventional polymers like polyethylene (PE) and polypropylene (PP), whose costs are between 0.23 and 0.48 dollars/Kg. Specifically, the highest cost lies in the substrates used to prepare culture media, like the carbohydrates needed to enrich these media, like glucose, sucrose, or fructose (Kumari-Bhuwal et al., 2013), constituting more than half of the total cost of BP production, reaching up to 70-80% of this cost (Mohanrasu et al., 2020). In this regard, agro-industrial wastes have been explored as alternatives for the elaboration of low-cost media, observing that some residues like fruit peels may represent feasible nutrient sources to support bacterial growth and promote high PHB production yields (Rivas-Castillo et al., 2022; 2023). To date, more than 300 different bacterial strains capable of producing PHB have been identified, such as *Bacillus cereus* ARY73 (Mohanrasu et al. 2020), *Klebsiella pneumoniae* E22 (Rivas-Castillo et al., 2022), and *Pseudomonas putida* (Mohanrasu et al. 2020). Among PHB producers, the genus *Bacillus* can be distinguished as it can accumulate more than 80% of its weight in PHB. In addition, *Bacillus* spp. are considered model organisms for research purposes, because they are abundant in nature, have high genetic plasticity and resistance to stressful conditions, molecular techniques are available to modify them, have a high growth rate and resistance to stressful conditions, and they can produce a wide variety of enzymes of industrial interest (Martínez-Herrera et al., 2023). Although *Bacillus* spp. are promising microorganisms for PHB production, their sporulating nature may be an interference, since spore formation requires the consumption of energy that can be obtained from PHB degradation, resulting in lower BP production yields (Madhusoodanan et al., 2022; Martínez-Herrera et al., 2023). PHB production with *B. megaterium* strain MNSH1-9K-1 has been previously studied in a medium prepared from orange-peel residues supplemented with complex carbon and nitrogen sources by a presence/absence experimental design approach, in which this production reached 112.62 g/L (Rivas-Castillo et al., 2023); however, these supplements are of complex nature and the specific growth conditions that may enhance this PHB production were not precisely defined. Therefore, the present work evaluated different growth conditions to determine the significance of diverse variables that influence this high production in the low-cost medium, to move forward into the knowledge needed to optimize this biotechnological process.

2. Methods

2.1 Preparation of the low-cost medium from orange-peel residues

Orange peels were collected in the municipality of Tizayuca, Hgo., Mexico. Peels were washed with running water and cut into pieces of approximately 2 cm, and subsequently dried at 60°C until constant weight. The culture medium was prepared following the methodology described in the patent application No. MX/a/2019/014627 of the Mexican Institute of Industrial Property (IMPI) (Rivas-Castillo et al., 2024).

2.2 Strain used and general growth conditions

B. megaterium strain MNSH1-9K-1 (GenBank accession number KM654562.1) was used for this study, which was isolated from a mining site in Guanajuato, Mexico (Rivas-Castillo et al., 2019). Pre-inoculums were prepared in 125-mL flasks containing 50 mL of nutrient broth (NB) at 150 rpm and 30°C for 24 h. Subsequently, cell growth was measured by total cell count using a Neubauer chamber, and experimental sets were performed as indicated for each case using also 125-mL flasks.

2.3 Experimental design to evaluate PHB production

A Taguchi experimental design with five central points was established to determine the effect of different physicochemical variables on PHB production; the variables considered were temperature, aeration (regulated by agitation in an orbital incubator and with the volume of the medium used in the flask), carbon (soluble starch), and nitrogen (sodium nitrate). The pH 6 and inoculums of 1×10^8 total cells were kept constant based on previous results (Rivas-Castillo et al., 2023). Table 1 shows the arrangement of the experimental design, consisting of 25 experimental sets (E1-E25). A control condition (C) was also included with the following conditions: 30°C, pH 7, 120 rpm, and 50 mL of medium ($n = 3$).

2.4 Extraction of PHB by the sodium hypochlorite-chloroform method

After 48 h of growth (Rivas-Castillo et al., 2024), each culture was centrifuged at 4000 rpm for 20 min. Then, the supernatant was removed and the accumulated PHB was extracted from the biomass (pellet) following the sodium hypochlorite-chloroform method (Rivas-Castillo et al., 2023).

2.5 Determination of carbon and nitrogen present in the culture medium

Carbon was determined based on the quantification of glucose, using the 3,5-dinitrobenzoic acid (DNS) method. Nitrogen was quantified by the vanadium chloride reduction technique. Quantifications were performed at 540 and 530 nm, respectively, using a UV-VIS spectrophotometer (DLAB, model SP-V1100) (Rivas-Castillo et al.,

2014). For each experimental condition, the C/N ratio was determined by considering the amount of carbon (glucose) and the total nitrogen present in the orange medium, plus the carbon and nitrogen added as soluble starch and sodium nitrate in each case. The following formula was used to calculate the equivalent percentage of carbon and nitrogen present in the added substrates (Chang-Feng et al., 2014):

$$\% C \text{ or } N = \frac{A}{B} \times 100$$

Where:

A = molecular weight of substrate

B = molecular weight of carbon or nitrogen

Table 1. Experimental sets evaluated based on the Taguchi experimental design.

Condition	Soluble starch (g/mL)	Sodium nitrate (g/mL)	Temperature (°C)	Volume (mL)	Agitation (rpm)	pH	Inoculum (total cells)
E1	0	0	30	50	30	6	1×10 ⁸
E2	0	0.1875	32.5	75	60	6	1×10 ⁸
E3	0	0.5	35	100	90	6	1×10 ⁸
E4	0	0.9375	37.5	125	120	6	1×10 ⁸
E5	0	1.5	40	150	150	6	1×10 ⁸
E6	2.5	0	32.5	100	120	6	1×10 ⁸
E7	3.125	0.3125	35	125	150	6	1×10 ⁸
E8	3.75	0.75	37.5	150	30	6	1×10 ⁸
E9	1.25	0.375	40	50	60	6	1×10 ⁸
E10	1.875	0.75	30	75	90	6	1×10 ⁸
E11	7.5	0	35	150	60	6	1×10 ⁸
E12	2.5	0.125	37.5	50	90	6	1×10 ⁸
E13	3.75	0.375	40	75	120	6	1×10 ⁸
E14	5	0.75	30	100	150	6	1×10 ⁸
E15	6.25	1.25	32.5	125	30	6	1×10 ⁸
E16	5.625	0	37.5	75	150	6	1×10 ⁸
E17	7.5	0.25	40	100	30	6	1×10 ⁸
E18	9.375	0.625	30	125	60	6	1×10 ⁸
E19	11.25	1.125	32.5	150	90	6	1×10 ⁸
E20	3.75	0.5	35	50	120	6	1×10 ⁸
E21	12.5	0	40	125	90	6	1×10 ⁸
E22	15	0.375	30	150	120	6	1×10 ⁸
E23	5	0.25	32.5	50	150	6	1×10 ⁸
E24	7.5	0.5625	35	75	30	6	1×10 ⁸
E25	10	1	37.5	100	60	6	1×10 ⁸

2.7 Statistical analyses

Basic statistical analyses such as arithmetic means, standard deviations, and analysis of variance (ANOVA), as well as the Taguchi experimental design and the Response Surface Model (RSM), were performed using the commercial software Minitab 19. Values with a $P \leq 0.05$ were considered as statistically significant.

1. 3. Results

3.1 Impact of physicochemical variables on PHB production

Figure 1 shows that, among the 25 experimental sets evaluated (E1-E25), plus the reference control (C), E25 was the condition that allowed the highest production yield, being of 106.47 g/L, under the following culture

conditions: 37.5°C, 60 rpm, 10 g of soluble starch, and 1 g of sodium nitrate in 100 mL of medium; under this experimental condition, a 4-fold increase in PHB production was achieved compared to C.

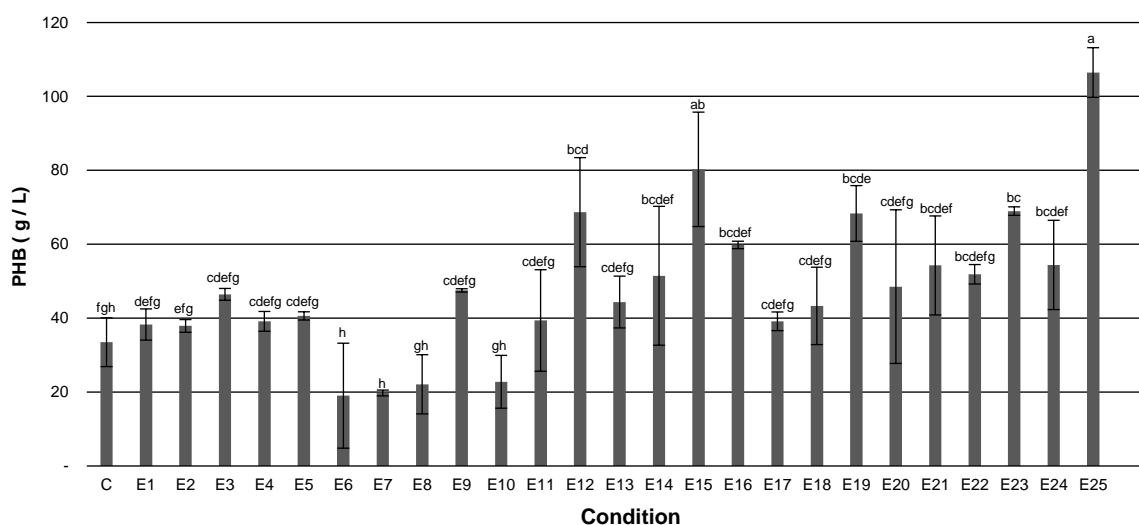


Figure 1. PHB production in the different conditions evaluated. C, control; E1-E25, different experimental conditions.

In addition, bacterial growth was evaluated by biomass production in each case, observing that the conditions with the highest biomass production were E24>E17>E13>E21>E14, which produced more than 110 g/L of biomass (data not shown). However, these conditions do not present the highest production yields; E25 had a lower biomass concentration (99 g/L) and presented the highest production. In addition, 18% of sporulation was shown in this experimental condition (E25), compared to C, which presented 89% of spores. The observed differences may be due to changes in the metabolism of *B. megaterium* due to the differences in the growth conditions exerted by the modifications in the physicochemical variables, such as C/N ratio or oxygen availability; as previously reported, it seems that more PHB is accumulated under stressful growth conditions (Rivas-Castillo et al., 2023, 2024). The C/N ratio observed in E25 was 16/1, highlighting the importance of the C/N relationship for promoting PHB production. Luvizetto-Faccin et al. (2013) and Mohanrasu et al. (2020) have emphasized that an adequate relationship between carbon and nitrogen in the culture medium is essential to promote PHB production by *B. megaterium*; as reported, the carbon source should be present in excess compared to nitrogen, to enhance high PHB production yields; if this does not happen, the carbon present will be only used for bacterial growth. For instance, when in some experimental conditions (like E1 to E5) no additional carbon source was added, cell growth was induced rather than PHB production (data not shown). Furthermore, the limitation of spore production could have induced PHB accumulation; previous reports by Brown et al. (1997) and Mohapatra et al. (2017) mentioned that the sporulation process, which is known to be generated by the limitation of nutritional sources, can be controlled with a C/N ratio suitable for the vegetative growth of the microorganism, consequently allowing the synthesis of PHB. In addition, an acidic pH of the medium may suppress sporulation. Regarding oxygen availability, aeration can be a fundamental factor in modulating PHB production, since it has been reported that low oxygen conditions influence the channel of nutrients, such as carbon, toward PHB synthesis (Madhusoodanan et al., 2022). Thus, low oxygenation, pH 6, and the C/N ratio 16/1 in E25 may have promoted the highest PHB production yield in the low-cost medium by directing the use of the saturating carbon source for BP production and simultaneously limiting spore formation.

3.2 Significance of physicochemical variables for PHB production

Based on the results obtained from the experimental design, a RSM was used to evaluate the effect of the physicochemical variables on PHB production, as well as the effect of their interactions on this response, by means of a fractional factorial model with central points (Table 3). Both the *F* and *P* values of the model were calculated by considering the mean squared values and the sum of squares. The descending order of the *F* values indicates the significance of the factors for the response variable, in addition to considering $P \leq 0.05$. All factors assessed were significant for the production process except for the interaction volume-rpm, whose $P > 0.05$. The value of $R^2 = 0.8973$ and $R^2_{adj} = 0.8272$ indicate that the model fits the experimental data reasonably well (Hamdy et al., 2022).

Complementarily, the Pareto diagram indicates the descendant significance of the variables for the response in terms of their absolute value, where those further from the adjustment line have a greater effect on PHB production. Thus, it was observed in accordance with F values, that the significance of the factors for PHB production is, in descending order: carbon > nitrogen > temperature > nitrogen-temperature > rpm > nitrogen-rpm > volume of the medium.

Table 3. Response Surface Model (RSM) for PHB production.

Factor	DF	Sums of squares	Mean square	F value	P value
Model	8	11399.50	1424.94	12.83	0 ^a
Lineal	5	9750.20	1950.03	17.55	0 ^a
A: Carbon	1	3601.40	3601.36	32.42	0 ^a
B: Nitrogen	1	3000.80	3000.84	27.01	0 ^a
C: Temperature	1	2266.00	2266.03	20.40	0 ^a
D: Volume	1	1193.80	1193.77	10.74	0.003 ^a
E: rpm	1	1547.30	1547.30	13.93	0.001 ^a
Interaction of 2 factors	3	2565.80	855.28	7.70	0.001 ^a
Nitrogen: Temperature (BC)	1	1692.50	1692.53	15.23	0 ^a
Nitrogen: rpm (BE)	1	1250.70	1250.73	11.26	0.002 ^a
Volume: rpm (DE)	1	241.50	241.46	2.17	0.151 ^b
Error	31	3444.10	111.10		
R ² (%)	89.37				
R ² _{adj} (%)	82.72				

^aSignificant

^bNon-significant

Overall, the results obtained suggest that it is possible to improve PHB production using a low-cost medium made from orange peels by its supplementation with additional substrates like soluble starch and sodium nitrate. The C/N ratio used, in addition to oxygen limitation and an acidic pH, seemed to modulate spore formation. These conditions can influence cellular metabolism, enhancing the expression of specific genes that codify for enzymes involved in the synthesis of the BP, so that the excessive carbon in the medium can be directed towards PHB synthesis (Quelas et al., 2016).

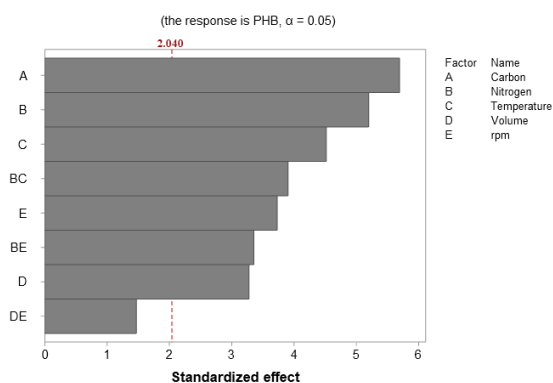


Figure 3. Pareto diagram of the significance of the physicochemical variables for PHB production.

It is relevant to mention that these growth conditions allowed the obtention of a similar production yield (106.5 vs 112.5 g/L) previously achieved in the same low-cost medium but complexly supplemented with eight substrates: soluble starch, sodium nitrate, sucrose, cane molasses, lactic casein, sodium acetate, and treated and untreated whey (Rivas-Castillo et al., 2023); then, it was possible to obtain a similar yield but with a likely diminishing in the costs of the medium needed to obtain a high PHB production yield, enhancing the future optimization of this biotechnological process to explore its scale-up feasibility.

4. Conclusion

The results obtained allowed the improvement of the PHB production process by *B. megaterium* strain MNSH1-9K-1 in a low-cost medium; the significance of the physicochemical variables involved was determined and an improved growth condition was established. It was observed the viability to produce PHB using a low-cost medium elaborated with orange peels, instead of using commercial media; the cost of producing the low-cost medium will be approximately 4.06 USD/L, while a medium prepared with reagent-grade substrates can cost 12.43 USD/L (Kumari-Bhuwal et al., 2013), which means a 33% decrease in production costs. Nevertheless, further studies are needed to enhance the process through an optimization approach.

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