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Improving PHB yield in *Halomonas halophila* through medium optimization

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Abstract

Polyhydroxyalkanoates (PHAs) offer a sustainable substitute for conventional petroleum-based plastics. Halophiles possess a remarkable ability to produce PHAs from low-cost by-products like crude whey or molasses, offering a sustainable alternative to traditional plastics. This study highlights the efficient production of PHAs by *Halomonas halophila* CCM 3662 using a basic culture medium designed to simultaneously enhance biomass and polyhydroxybutyrate (PHB) production. Through optimization via response surface methodology (RSM), the optimal medium composition was identified as 22.85 g/l glucose, 4.36 g/l ammonium sulphate, and 13.22 g/l sodium hydrogen. Under these conditions, biomass production reached 1.44±0.07 g/l, while PHB content achieved 50.16±0.84%. This represented a 2.8-fold increase in biomass production and a 2.5-fold increase in PHB production compared to a medium without optimized composition. This study provides valuable insights into the optimal medium composition for the growth of the halophilic *H. halophila* and its potential application in PHB production for various uses.

Introduction

The growing interest in developing biodegradable plastics in recent years is largely motivated by shifting climate conditions and the need to mitigate the environmental impact of petroleum-based, non-biodegradable plastics (Demirbas 2007; Folino *et al*. 2020; Kibria *et al*. 2023; Mangal *et al*. 2023). Polyhydroxyalkanoates (PHAs) are among the potential candidates for replacing conventional plastics. PHAs are carbon-neutral and technologically valuable polymers produced by microorganisms from various renewable carbon sources, making them a sustainable and environmentally friendly material. While numerous bacterial species are known to produce these polymers (Chmelová *et al*. 2020; Chmelová *et al*. 2020; Rondošová *et al*. 2022), halophilic bacteria have also garnered interest. Halophiles represent a phylogenetically diverse group, but they share a common characteristic: they prefer or even require, a hypersaline environment for optimal growth, with sodium chloride typically serving as the primary salt component.

Members of the moderately halophilic group of bacteria not only tolerate NaCl but require it as an essential component of their culture medium. This group includes *Halomonas* spp. such as

H. boliviensis, *H. campaniensis*, *H. elongata*, *H. salina*, *H. profundus*, *H. venusta*, *H. neptunia*, *H. hydrothermalis*, *H. marina*, *H. smyrnensis*, *H. pacifica* and *H. salifodiane*. One noteworthy member of this group is *Halomonas halophila* (previously known as *Deleya halophila*), a Gram-negative, strictly aerobic, and moderately halophilic bacteria originally isolated in southeastern Spain (Quesada *et al*. 1984). *H. halophila* has shown the ability to produce polyhydroxybutyrate (PHB) from variety of low-cost substrates, including whey hydrolysates, coffee processing residues, sawdust, and corn straw. Such substrates are typically treated as waste in the primary sector of the economy, highlighting the industrial potential of *H. halophila* for PHB production (Kucera *et al*. 2018; Kovalcik *et al*. 2018; Kovalcik *et al*. 2020). Additionally, lignocellulose-based waste materials have also been identified as suitable feedstocks for PHB production by this producer (Kucera *et al*. 2018; Kourilova *et al*. 2021). This versatility in utilizing inexpensive and renewable substrates makes *H. halophila* a promising candidate for sustainable PHA production. However, to fully harness its potential, is critical to establish a basic culture medium composition that optimally supports both biomass growth and PHB accumulation. The aim of this work was to optimize the composition of a basic culture medium for *H. halophila* CCM 3662 using response surface methodology (RSM). This optimized medium could serve as a foundation for further studies, enabling the replacement of the carbon source with other inexpensive alternatives, ideally derived from the primary sector of the economy and otherwise considered waste. To the best of our knowledge, no optimization of the culture medium composition for PHB production by *H. halophila* has been conducted to date.

Experimental

Microorganism

Halomonas halophila CCM 3662 was purchased from the Czech Collection of Microorganisms of the Institute of Experimental Biology, Masaryk University (Brno, Czech Republic). *H. halophila* was initially cultured on Nutrient Agar No. 2 supplemented with 1% (w/v) agar and sodium chloride at a concentration of 66 g/l. The propagation medium, composed of peptone (15 g/l), yeast extract (3 g/l), glucose (1 g/l), and NaCl (66 g/l), was inoculated with a glycerol stock culture. After 24 hours of cultivation, this propagated culture was used to inoculate the basic culture medium at a 3% (v/v) inoculum ratio.

Basic culture medium composition

The basic culture medium was adapted from the other studies (Kucera *et al*. 2018; Kovalcik *et al*. 2018; Kourilova *et al*. 2021) and contained glucose (20 g/l), $(NH_4)_2SO_4$ (3 g/l), KH_2PO_4 (1 g/l), Na_2HPO_4 .$12H_2O$ (11.1 g/l), $MgSO_4$.$7H_2O$ (0.2 g/l), NaCl (66 g/l) a solution with trace elements (1 ml/l) ($FeCl_3$ (9.7 g/l), $CaCl_2$ (7.8 g/l), $CuSO_4$.$5H_2O$ (0.156 g/l), $CoCl_2$ (0.199 g/l), $NiCl_2$ (0.118 g/l) dissolved in HCl (0.1 mol/l)). After an initial Plackett-Burman analysis (data not shown) of the necessity of the presence of each component, the medium was modified as follows: glucose (20 g/l), $(NH_4)_2SO_4$ (3 g/l), Na_2HPO_4 .$12H_2O$ (11.1 g/l), $MgSO_4$.$7H_2O$ (0.2 g/l) a NaCl (66 g/l). The pH of the medium was set to 7.0. We varied the composition of the medium components with significant effect on bacterial growth, namely glucose in the concentration range of 5 - 50 g/l, ammonium sulphate in the concentration range of 1 - 10 g/l, sodium hydrogen phosphate dodecahydrate in the concentration range of 1.5 - 20 g/l and sodium chloride in the concentration range of 10 - 70 g/l. Cultivation was carried out for 72 hours, dynamically (min. 150 RPM) at 30°C and pH 7.0.

Cultivation medium optimization

The composition of the culture medium was optimized by the response surface methodology (RSM). Glucose concentration, ammonium sulphate concentration and sodium hydrogen phosphate concentration were chosen as independent variables. The dependent variables were the biomass yield (g/l) and the PHB yield (%). The three independent variables were tested at five coded values, namely -1.682; -1; 0; 1 and 1.682 (Table 1).

Table 1: Interpretation of coded levels of the three independent variables tested by RSM.

Variables	Code levels				
	-1.682	**-1**	**0**	**1**	**1.682**
Glucose concentration (g/l)	8.63	12	17	22	25.37
Ammonium sulphate concentration (g/l)	1.32	2.5	4.25	6.0	7.18
Sodium hydrogen phosphate concentration (g/l)	4.31	7	11	15	17.69

The second-order polynomial function with respect to the three selected parameters is given by Equation (1).

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2} b_{ij} X_i X_j \quad (1)$$

where X are the independent variables (glucose concentration, ammonium sulphate concentration or sodium hydrogen phosphate concentration), Y is the response (biomass yield and PHB yield), and b are the regression coefficients (b_i – the linear coefficients; b_{ii} – the quadratic coefficients; b_{ij} – interaction coefficients).

Isolation and PHA determination

After 72 hours of cultivation, the culture medium was centrifuged at 4,000 RPM for 20 minutes, and the *H. halophila* biomass was dried to a constant weight at 50°C. The dried biomass was then mixed with chloroform at a ratio of 1:100 (w/v), and the extraction was carried out at laboratory temperature for 24 hours. The extract was dried with anhydrous sodium sulphate, filtered, and evaporated. The sediment was quantified as crude PHA yield (%) relative to the biomass. Subsequently, the crude PHAs was dissolved in chloroform, depolymerized, derivatized and analyzed using gas chromatography with a flame ionization detector (GC-FID).

Determination of PHB yield

The crude PHAs were dissolved with chloroform (2 ml). To this solution, acid methanol (6% (v/v)) was added (2 ml), and the mixture was depolymerized and derivatized at 100°C for 1 hour. After cooling, 1 ml of distilled water was added, and the mixture was allowed to stand for 24 hours. Following this, 1 ml of the lower chloroform phase was carefully pipetted off and determined by GC-FID (6890N, Agilent Technologies, USA). A sample (2 µl) was injected, with the injection valve maintained at 250°C, and the split ratio set to 100:1. The sample was separated on an RTX-1 column under a pressure of 43 kPa and a carrier gas flow rate of 1.1 ml/min (nitrogen). After injection, the column temperature was held at 80°C for 1 minute, then increased at a rate of 10°C/min up to 150°C. Once this temperature was reached, the temperature was increased at 20°C/min to 280°C and held for an additional 5 minutes. The FID was set to 270°C. The PHB yield was quantified using a calibration curve based on the known concentration of 3-hydroxybutyrate (3-HB), and the result was expressed as % relative to the dry biomass of *H. halophila* CCM 3662.

Statistical methods

The data analysis was performed using Microsoft Excel (2019), with all experiments and cultivations conducted in triplicate. Optimization was carried out using RSM in Statgraphics (The Plains, Virginia, USA).

Results and discussion

The composition of the culture medium plays a critical role in PHA production by *H. halophila* (Obruca *et al*. 2015; Kucera *et al*. 2018). The initial medium composition was based on a thorough review of the literature on PHA production by *H. halophila* (Kucera *et al*. 2018; Kovalcik *et al*. 2018; Pernicova *et al*. 2019; Kourilova *et al*. 2021). Before optimizing the medium using RSM, it was essential to understand the individual effects of various factors on biomass production, as well as on PHA and PHB yields. From prior Plackett-Burman analysis (data not shown), we identified several key components, namely glucose (carbon source), ammonium sulphate (nitrogen source), sodium hydrogen phosphate, and sodium chloride, as having a significant impact on both *H. halophila* biomass and PHB production. Based on these findings, the medium composition was modified to include five key components: glucose, ammonium sulphate, sodium hydrogen phosphate, NaCl, and

magnesium sulphate. In the subsequent experiments, we varied the concentrations of each component and evaluated their effects based on the resulting biomass yield (Figure 1).

The highest biomass yield was achieved in the medium containing 15 - 20 g/l glucose (Figure 1a). This trend aligns with findings from several published studies, which report that suitable glucose concentrations significantly influence growth and biomass production in halophilic bacteria (Obruca *et al*. 2014; Kucera *et al*. 2018). For ammonium sulphate concentration, the highest biomass was observed at an initial concentration of 1 g/l (Figure 1b), with this value (1.27 g/l) being comparable to the maximum biomass yield seen with varying glucose concentrations. At higher concentrations of ammonium sulphate, a decreasing trend in biomass production was noted. This suggests an inverse relationship, the higher the ammonium sulphate concentration, the lower the biomass produced by *H. halophila*. Although this result may seem unexpected, some studies (Aldén *et al*. 2001; Zhang *et al*. 2018) suggest that excessive nitrogen concentrations can inhibit growth and biomass production in certain bacterial species. In some cases, nitrogen limitation can promote biomass production. When nitrogen becomes a limiting nutrient, bacteria may prioritize cell growth over other metabolic processes, leading to enhanced cell division and biomass accumulation. This is likely due to more efficient use of available carbon resources to support growth. Additionally, nitrogen limitation can induce shifts in cellular metabolism and gene expression. At ammonium sulphate concentrations higher than 10 g/l, no further increase in biomass production was observed (Figure 1b), possibly due to the depletion of other essential components in the medium required for growth.

When evaluating the impact of sodium hydrogen phosphate concentration, results reveal an increasing trend up to a concentration of 11.1 g/l (Figure 1c). At higher concentrations, sodium hydrogen phosphate can become toxic to bacterial cells. Excessive phosphate ions may disrupt cellular processes, inhibiting bacterial growth. As a result, high levels of sodium hydrogen phosphate in the culture medium could hinder bacterial growth. Mohan and Reddy (2013) observed that phosphate ion deficiency reduces the rate of protein synthesis, ultimately affecting biomass production. Moreover, sodium hydrogen phosphate acts as a buffering agent, helping to stabilize the pH of the culture medium. Like all bacteria, *H. halophila* has specific pH requirements for optimal growth. Maintaining the pH within an appropriate range is crucial for providing suitable environmental conditions. In our case, despite the absence of acidic phosphates in the culture medium, *H. halophila* did not produce significant amounts of substances that would substantially alter the pH. Starting with an initial pH of 7.0, the pH of the medium gradually decreased, depending on its composition, to values between 6.5 and 6.8 after 72 hours of cultivation (data not shown). Regarding sodium chloride, it is evident that a concentration of 10 g/l was insufficient, while the highest biomass yield occurred in the medium containing 25 g/l NaCl (Figure 1c). This result is not surprising, as higher salt concentrations are necessary for the growth of halophilic bacteria like *H. halophila*. Elevated salinity affects various aspects of bacterial physiology and metabolism. These adaptations include the accumulation of osmoprotectants, such as ectoine, to maintain osmotic balance, alterations in membrane structure to resist osmotic stress, and changes in enzyme activity and gene expression to support growth and survival in saline environments (Brown *et al*. 1986; Kates 1986; Adams and Russel 1992; Cummings and Gilmour 1995; Hobmeier *et al*. 2022).

Figure 2 presents the results from the same experiment, showing the changes in the basic culture medium components in relation to the amounts of PHA and PHB produced. *H. halophila* produced the highest amount of PHB at the highest glucose concentration of 50 g/l (Figure 2a). Typically, PHB production in bacteria is triggered under conditions of nutrient limitation or excess carbon availability. When nutrients such as nitrogen or phosphorus are scarce, bacteria may alter their metabolic pathways to store excess carbon in the form of PHB for future energy needs. Additionally, PHB production is influenced by various factors, including growth conditions such as nutrient availability, pH, temperature, oxygen levels, and genetic traits (McAdam *et al*. 2020).

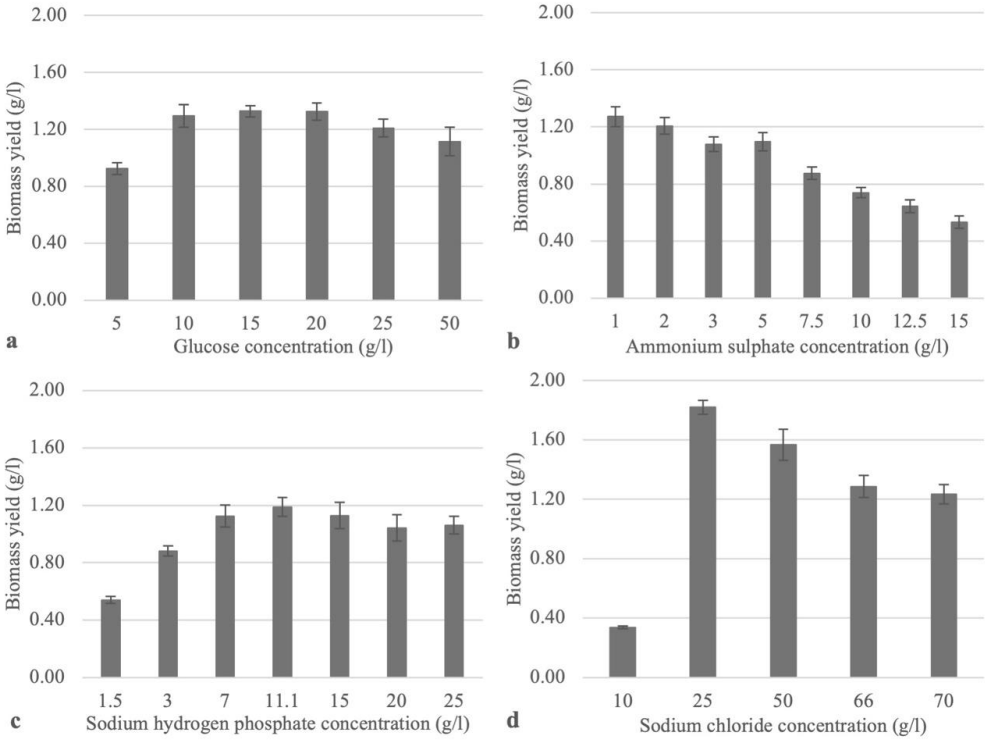

Figure 1: Biomass yield (g/l) of *H. halophila* in media with varying concentrations of glucose (a), ammonium sulphate (b), sodium hydrogen phosphate (c), and sodium chloride (d).

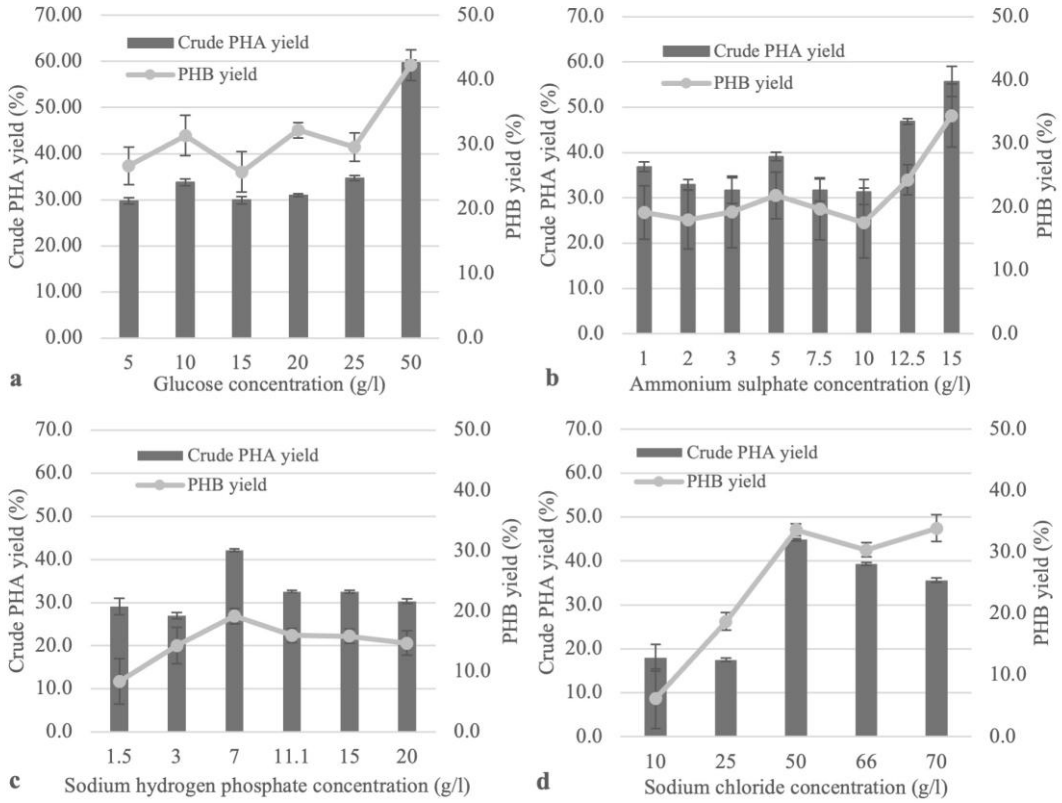

Figure 2: Crude PHA yield (%) and PHB yield (%) in media with varying concentrations of glucose (a), ammonium sulphate (b), sodium hydrogen phosphate (c), and sodium chloride (d).

The PHB content ranged from 25.7 to 42.3% (Figure 2a). Within the standard deviation error margin, the PHB content remained relatively stable up to an ammonium sulphate concentration of 10 g/l. Interestingly, however, the highest PHB yield (34.4±5.0%) was observed at the highest ammonium sulphate concentration of 15 g/l. It is generally known that PHB production in bacterial bioplastics producers is stimulated by high concentrations of carbon sources and, conversely, by low nitrogen concentrations. In media designed for *H. halophila* cultivation targeting PHB production, ammonium sulphate is typically used at a concentration of 3 g/l (Kucera *et al*. 2018; Kovalcik *et al*. 2018; Kourilova *et al*. 2021). However, Shahid *et al*. (2013) reported a similar finding for PHB production using *Bacillus megaterium* DSM 90 and *Pseudomonas putida* mt-2. In their study, analysis of whole-cell spectra by FTIR enabled the rapid identification of significant PHA producers and showed that nitrogen depletion is not always necessary for PHA synthesis, provided glucose is available at an appropriate concentration. This trend was later confirmed by GC analyses on four selected bacterial strains, where PHA accumulation was observed. Therefore, the requirement for nitrogen limitation in PHA production depends on the microorganism and/or the concentration of the available carbon substrate.

The highest PHB yield was measured in the medium with the highest concentration of sodium hydrogen phosphate (20 g/l) (Figure 2c), where PHB yield ranged from 8.4 to 19.2%. From Figure 2d, it is evident that a sodium chloride concentration of 10 g/l was insufficient for PHB production (6.2±4.8%). In media with varying NaCl concentrations, PHB yields ranged from 6.2 to 33.9%. Interestingly, while the suitable sodium chloride concentration for biomass production was approximately 25 g/l (Figure 1d), concentrations exceeding 50 - 70 g/l were more suitable for enhancing PHB production (Figure 2d). This suggests that high NaCl concentrations could be employed to stimulate PHB production after the initial biomass production, following an appropriate time interval.

Table 2 Experimental matrix with actual coded level of independent variables and measured values of biomass yield (g/l) and PHB yield (%).

Exp.	Glucose concentration (g/l)	Ammonium sulphate concentration (g/l)	Sodium hydrogen phosphate concentration (g/l)	Biomass yield (g/l)	PHB yield (%)
1	12	2.5	7	0.85±0.00	37.7±0.00
2	22	2.5	15	1.04±0.31	22.2±0.00
3	22	6	7	0.57±0.02	46.4±1.27
4	17	4.25	11	1.26±0.04	51.0±2.44
5	12	6	15	1.07±0.06	44.8±1.82
6	22	2.5	7	1.11±0.19	44.6±6.40
7	22	6	15	1.17±0.11	52.4±0.00
8	12	2.5	15	0.15±0.11	8.7±0.00
9	17	4.25	11	1.54±0.18	48.8±1.30
10	12	6	7	0.93±0.03	51.4±0.00
11	25.37	4.25	11	1.20±0.05	48.4±0.66
12	17	7.18	11	0.77±0.13	52.5±1.30
13	17	4.25	11	1.15±0.17	43.6±0.00
14	8.63	4.25	11	0.734±0.11	8.4±0.36
15	17	4.25	4.31	0.77±0.04	46.1±1.67
16	17	4.25	17.69	0.76±0.01	24.5±4.21
17	17	1.32	11	0.00±0.00	0.0±0.00

Optimization of basic culture medium composition for Halomonas halophila

Based on these results, we selected the following independently variables, namely glucose concentration in the range of 12 - 22 g/l, an ammonium sulphate concentration in the range of 2.5 - 6.0 g/l, and a sodium hydrogen phosphate concentration in the range of 7 - 15 g/l. The sodium chloride concentration was set at a fixed value 25 g/l. This choice was because higher NaCl concentrations did not stimulate PHB production (Figure 2d), but not biomass production (Figure 1d). As a compromise between these two crucial factors, we chose for a NaCl concentration of 25 g/l. Both biomass production and PHB yield determined by GC-FID, were chosen as the dependent variables. The results are summarized in Table 2.

The highest biomass production was observed at a glucose concentration of 17 g/l, ammonium sulphate at 4.25 g/l, and sodium hydrogen phosphate at 11 g/l, yielding 1.54±0.18 g/l of *H. halophila* biomass. These conditions also enabled the accumulation of significant amounts of PHB, ranging from 43.6 to 51.0% (Table 2). The results suggest that optimizing the concentration of various culture medium components can stimulate both biomass production in *H. halophila* and the accumulation of PHB. No growth was observed in the medium with a limiting ammonium sulphate concentration of 1.3 g/l, and similarly low biomass production occurred at the lowest glucose concentration used, 12 g/l. The highest PHB yield (52.5±1.30%) was observed in biomass cultured in medium containing 17 g/l glucose, 7.18 g/l ammonium sulphate, and 11 g/l sodium hydrogen phosphate (Table 2). These findings support the conclusions drawn from the optimization threshold analysis, where higher ammonium sulphate concentrations stimulated PHB production (Figure 2). Except for experiment 17, the lowest PHB yields were observed in media with low glucose concentrations (12 and 8.6 g/l), producing 8.7±0.00% and 8.4±0.36% PHB, respectively.

From the obtained data, we calculated the coefficients for the second-degree polynomial equations. The coefficients of determination (R^2) were 94.76% for biomass yield and 92.02% for PHB yield. To better visualize the interactions between the selected variables, we used the standardized Pareto graphs (Figure 3), which depict the effects of glucose concentration, ammonium sulphate, and sodium hydrogen phosphate on the dependent variables: biomass yield (Figure 3a) and PHB yield (Figure 3b).

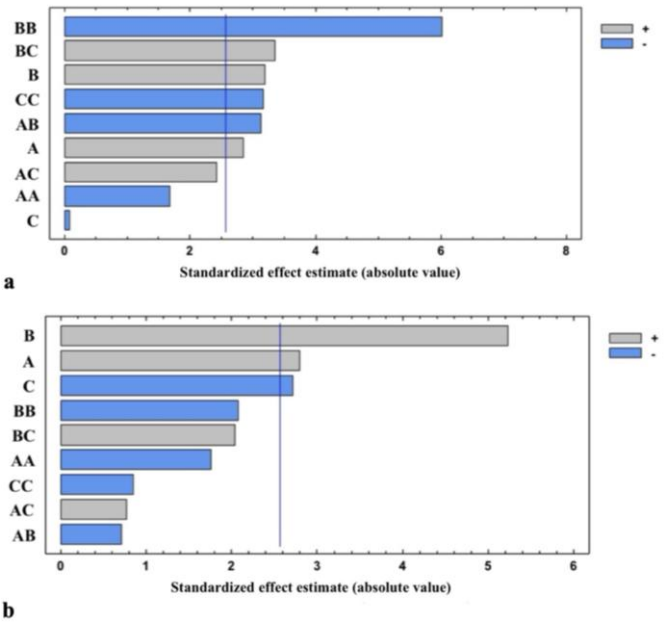

Figure 3: Standardized Pareto Chart for biomass yield (a) and PHB yield (b). Positive influences are shown in grey and negative influences on the observed dependent variables are shown in blue (A – glucose concentration; B – ammonium sulphate concentration a C – sodium hydrogen phosphate concentration).

The quadratic effects of ammonium sulphate (BB) and sodium hydrogen phosphate (CC) concentrations in the basic culture medium had a significantly negative impact on biomass yield (Figure 3a). This suggests that, at lower concentrations of nitrogen or phosphorus, the decrease in biomass yield is more gradual, whereas at higher concentrations, the decline becomes more pronounced, as commonly observed with exponential functions. The concentrations of carbon (glucose) and nitrogen (ammonium sulphate) exhibited a positive linear effect on biomass production, meaning that biomass increased as the concentrations of these variables (Figure 3b). Among the interaction effects, the relationship between nitrogen and phosphorus (BC) concentrations showed a positive interaction,

where increasing the concentrations of both variables resulted in higher biomass production. In contrast, the interaction between carbon and nitrogen concentrations (AB) had a negative effect on biomass quantity. All the aforementioned variables significantly impacted biomass abundance, either positively or negatively.

For PHB production, the effect was statistically significant only for carbon (A), nitrogen (B), and phosphorus (C) concentrations. Both carbon and nitrogen concentrations had a positive linear effect on PHB yield, while sodium hydrogen phosphate concentration had a negative linear effect, meaning that PHB production decreased with higher concentrations of sodium hydrogen phosphate (Figure 3b). The data suggest that the composition of the basic culture medium can be adjusted to stimulate both biomass and PHB production, as indicated by the positive linear effects of both glucose and ammonium sulphate concentrations on the yield of both biomass and PHB. These findings are further supported by the regression coefficients presented in Table 3.

From the results in Table 3, we observed that six effects have *p*-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. For PHB yield, three effects showed *p*-values below 0.05.

Model optimization and verification

The predicted values calculated from the model reveal that the optimal conditions for maximizing biomass production differ from those for maximizing PHB production (Table 4).

Maximum biomass production of *H. halophila* can be achieved in a culture medium containing 22.85 g/l glucose, 4.36 g/l ammonium sulphate, and 13.22 g/l sodium hydrogen phosphate (Table 4).

Table 3: Regression coefficients of the predicted second-degree polynomial model for biomass and PHB yield.

Effect	Variables	Biomass yield (g/l)	PHB yield (%)
Constant		-2.116	-43.9037
Linear	A: Glucose concentration	**0.1443**	**7.4073**
	B: Ammonium sulphate concentration	**0.9221**	**16.6882**
	C: Sodium hydrogen phosphate concentration	-0.0228	**-4.4219**
Quadratic	AA	-0.0032	-0.1857
	BB	**-0.0938**	-1.790
	CC	**-0.0095**	-0.1406
Interaction	AB	**-0.0201**	-0.2543
	AC	0.0068	0.12
	BC	**0.0270**	0.9071

*Effects with a *p*-value less than 0.05 are highlighted in bold.

Table 4: Optimization conditions by RSM and predicted and observed values of dependent variables.

Independent variables					
Glucose concentration (g/l)	**Ammonium sulphate concentration (g/l)**	**Sodium hydrogen phosphate concentration (g/l)**	**Dependent variables**	**Predicted value**	**Observed value**
22.85	4.36	13.22	**Biomass yield (g/l)**	1.39	1.44±0.07
20.22	7.18	16.06	**PHB yield (%)**	56.85	55.19±2.4

Under these optimal conditions, the predicted biomass concentration is 1.39 g/l. However, to achieve the maximum PHB yield, only the glucose concentration needs to be slightly adjusted to 20.22 g/l, while the nitrogen and phosphorus concentrations must be increased to 7.18 g/l and 16.06 g/l, respectively. Under these conditions, up to 56.85% PHB can be produced. In both

scenarios, the model validation achieved values close to those predicted, namely 1.44±0.07 g/l biomass yield and 55.19±2.4% PHB yield. This indicates that the model is well-designed and accurately describes the effects on *H. halophila* biomass growth and PHB accumulation.

Given that the aim of this study was to design a basic culture medium that stimulates biomass production without inhibiting PHB production, the optimized medium for maximizing biomass content is recommended. This medium contains 22.85 g/l glucose, 4.36 g/l ammonium sulphate, 13.22 g/l sodium hydrogen phosphate, and 25 g/l sodium chloride. Under these conditions, the PHB content per 1.44±0.07 g/l *H. halophila* biomass was 50.16±0.84% (data not shown). The basic composition of the medium was based on a review of literature focusing on PHB production by *H. halophila* (Kucera *et al*. 2018; Kovalcik *et al*. 2018; Pernicova *et al*. 2019; Kourilova *et al*. 2021). For *H. halophila* CCM 3662, the concentration of ammonium sulphate and sodium hydrogen phosphate was increased from the original 3 g/l and 11.1 g/l to 4.4 g/l and 13.1 g/l, respectively. Additionally, potassium dihydrogen phosphate, magnesium sulphate, and trace element solutions were not necessary for biomass or PHB production. Compared to the initial medium composition (data not shown), the optimized medium led to an increase in biomass from 0.52 g/l to 1.44±0.07 g/l and PHB yield from 19.91% to 50.16±0.84%.

Conclusions

We focused on optimizing the efficient production of PHB using *H. halophila* CCM 3662 in a basic culture medium using RSM. The maximum biomass production of *H. halophila* was achieved in a medium containing 22.85 g/l glucose, 4.36 g/l ammonium sulphate, and 13.22 g/l sodium hydrogen phosphate, with a fixed sodium chloride concentration of 25 g/l. Upon validating the results, the suitability of the model used was confirmed. This work provides valuable insights into the optimal culture conditions for the halophilic bacteria *H. halophila*, supporting the potential for biopolymer production in various biotechnological applications.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this study the authors used ChatGPT in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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