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Optimal Medium Composition to Enhance Poly-β-hydroxybutyrate Production by *Ralstonia eutropha* Using Cane Molasses as Sole Carbon Source

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Abstract

In order to reduce the costs associated with poly-β-hydroxybutyrate production, growth and poly-β-hydroxybutyrate production of *Ralstonia eutropha* were studied in batch culture on different carbon sources. Experiments were designed and conducted to not only lower the cost of poly-β-hydroxybutyrate production by using inexpensive substrates, but also to increase poly-β-hydroxybutyrate production by optimizing the culture medium composition. Low cost, abundant carbon sources, including cane molasses, beet molasses, soya bean, and corn steep liquor were used to investigate the possibility of poly-β-hydroxybutyrate production in such renewable carbon sources. Based on the experimental results, cane molasses with production of 0.49 gL^{-1} poly-β-hydroxybutyrate was selected as the most efficient carbon source. To improve bacterial growth and poly-β-hydroxybutyrate production, different chemicals were then used to pretreat the cane molasses. Sulfuric acid, with 33% enhancement in poly-β-hydroxybutyrate production, revealed the highest efficiency in removing heavy metals and suspended impurities and was used to pretreat cane molasses in the subsequent experiments. Additionally, to make the process even more efficient and ultimately more effective, urea and corn steep liquor were used as nitrogen/minerals and vitamin sources, respectively. Using the response surface methodology and through a 2^n factorial central composite design, the medium composition was then optimized, and maximum biomass concentration of 5.03 gL^{-1} and poly-β-hydroxybutyrate concentration of 1.63 gL^{-1} were obtained.

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1. Introduction

As a consequence of industrial growth, accumulation of non-degradable waste materials, such as petroleum based plastics, in natural environments has become one of the most challenging issues during the last decades. It takes many decades for conventional plastics to be broken down in nature [1, 2]. However, even after such long period, decomposition of petroleum based polymers results in toxins, which can negatively affect the ecosystem health and function [3]. In recent years, there is an increasing interest in utilizing degradable materials that can be easily decomposed to harmless elements after relatively quick decomposition [4, 5] and thus, considerable effort has been devoted to develop microbial based

technologies to generate biomaterial and biofuels [3, 6, 7].

In response to physicochemical stresses, a large variety of prokaryotic and eukaryotic microorganisms synthesize and accumulate biopolymers such as Poly-β-hydroxybutyrate (PHB), as intracellular carbon/energy storage compounds [8, 9]. For example, it has been reported that the absence (i.e. presence in suboptimal concentrations) of either macro-elements (i.e. H, N, P, or O) or particular micro-elements (i.e. Ca, Mg, etc.) can promote bacterial PHB production [10-12]. Complete biodegradation of PHB has been observed in soil, water, and swage to either water-carbon dioxide or methane under aerobic and anaerobic conditions, respectively [13]. In addition to biodegradability, PHB is a non-toxic thermoplastic that can be produced by microorganisms using renewable resources. Such characteristics have made PHB an environmentally sustainable alternative to petrochemical derived polymers [4, 5, 14, 15]. However, currently, production cost of PHB prevents wider adoption and makes it noncompetitive with the petrochemical based plastics [14, 16]. The high production cost of PHB can be explained by narrow microbial PHB synthesis in compare to the large economic scale. Cost of the raw materials, as well as the fermentation and downstream processes to produce, extract, and isolate PHB are main factors contributing to the high cost of industrial PHB manufacturing. Therefore, improved media composition and cultivation methods [12, 17], as well as more efficient downstream processes [16, 18, 19] have been developed to make PHB production economically viable and commercially acceptable.

Much of the operating cost of PHB production is attributable to utilization of expensive raw materials as production substrates [4, 8, 20]. Thereby, low cost and relatively abundant nutrient sources such as pulp industry waste [21], dairy waste [22], sugar industry residues [23], C1 carbon resources including methane and CO_2 [24], and other agro-industrial wastes [19,25, 26] have been used to make production of such high valued biopolymers both environmentally, and economically favorable.

As a byproduct of sugar industry, cane molasses consists of water, sucrose, nitrogen, proteins, vitamins, amino acids, organic acids, and metal ions. Therefore, molasses can strongly support bacterial growth and biological activities, and can in effect substitute for a wide range of nutrients in culture media. However, due to the high concentration of heavy metals and suspended impurities, supplementing culture medium with cane molasses may be detrimental to microorganisms by altering the medium pH and inactivating the enzymes associated with biosynthesis of products [26-28]. For instance, high concentration of heavy metals has been reported to be responsible for negligible accumulation of PHB in bacteria growing in agro-industrial wastes [26].

In this study, using *R. eutropha*, PHB production was investigated in culture media composed of different domestic low cost and abundant carbon sources. Also, instead of costly synthetic substances such as yeast extract [12, 29], Corn Steep Liquor (CSL) was used as a source of vitamins and growth factors to make the process even more economic. Subsequently, as one of the most commonly used methods to evaluate optimum culture media composition [12, 30, 31], response surface methodology (RSM) was used to assess the optimal concentration of nutrient sources to enhance PHB production in shake flask cultures.

2. Materials and Methods

2.1. Microorganism

Strong capability in accumulating PHB from simple carbon sources has made *Ralstonia eutropha* the most prominent and most widely used PHB accumulating bacterium [12, 20, 25, 32]. Therefore, to produce PHB, *Ralstonia eutropha* PTCC1615 was obtained from Persian Type Culture Collection (Iran), and used throughout the experiments. As recommended by Persian Type Culture Collection, LB Agar (10.0 gL^{-1} Peptone, 5.0 gL^{-1} Yeast extract, 5.0 gL^{-1} NaCl with 1.5% agar) was used to maintain and expand cell population. The stock cultures were maintained on LB Agar slants at 4°C and subcultured monthly.

2.2. Culture media

The LB broth media (pH=7.0) was used to prepare the inocula. Cells harvested from stock cultures were transferred to a 250 mL Erlenmeyer flask with 100 mL of LB broth. The flasks were incubated at 30°C for 24 h in a rotary shaker incubator (Labcon 5082U, South Africa) at 150 rpm and used as inoculum (5% vv^{-1}) in the experiments. Prior to inoculation, Sudan black staining method [33] was used to confirm capability of bacteria in producing PHB.

The mineral salt medium consisting of 2.0 gL^{-1} KH_2PO_4 and 0.2 gL^{-1} $MgSO_4.7H_2O$ was used throughout the experiments to prepare culture media. Based on availability and cost, cane molasses (Center of Iranian Development of Cane Industry), beet molasses (Gazvin Sugar Manufacturing Comp-any), soya bean (Falahat Pishe Kordkuy, Gorgan-dane), and CSL (Glucosan Company) were selected as four different carbon sources. For consistency, in each experiment, the mineral salt medium was supplemented with 10% of each selected carbon source. However, although different carbon sources were utilized, in all the experiments, urea (2 gL^{-1}) and CSL (1 gL^{-1}) were used as nitrogen and vitamin sources. The pH of the resulting media were adjusted

to 7.0 with 1 N NaOH/1 N HCl, sterilized by autoclaving at 121°C for 20 min, and then cooled down at room temperature prior to inoculation. To study PHB production, 100 mL of the medium was transferred to a 250 mL Erlenmeyer flask and then inoculated and cultivated in the shaker incubator (150 rpm) at 30°C for 120 h. At regular intervals, samples were withdrawn and analyzed for biomass and PHB concentrations. All the experiments were conducted in triplicate.

2.3. Soya bean extraction

Dried and ground soya beans were submitted to soxhlet extraction for 8 h by hexane at 70°C (solvent boiling temperature) [34]. To evaporate the solvent, the obtained solution containing soya bean extract was placed in an evaporator at 30°C, and the remaining oil was used as soya bean extract in the experiments.

2.4. Pretreatment of cane molasses

Cane molasses was obtained from the Center of Iranian Development of Cane Industry. It was diluted with distilled water in order to obtain 10% wv^{-1} total molasses. The molasses solutions were used after being chemically treated [27, 28].

2.4.1. Sulphuric acid treatment

The pH of molasses solution was adjusted to 3.0 with 1 N H_2SO_4. The liquid was allowed to stand for 24 h, and then centrifuged at 5000 g for 25 min. The pH of the supernatant was then adjusted to 5.5 with 1 N NaOH, and sterilized at 121°C for 20 min [28].

2.4.2. Tricalcium phosphate treatment

The molasses solution was adjusted to pH 7.0 with 1 N HCl and heated at 100°C for 15 min. The liquid was then treated with 0.03 M tricalcium phosphate while the temperature was maintained between 80°C and 90°C. The mixture was allowed to stand for 24 h at room temperature and then centrifuged at 5000 g for 25 min. The pH of the supernatant was adjusted to 5.5 with 0.1 N HCl and sterilized at 121°C for 20 min. The insoluble matter was then separated by centrifugation at 5000 g for 15 min [28].

2.4.3. EDTA treatment

The molasses solution was adjusted to pH 5.5 with 5 N HCl and heated at 100°C for 15 min. By keeping the temperature between 80°C and 90°C, 100 ppm of EDTA was added to encourage precipitation of heavy metals. The mixture was allowed to stand for 24 h at room temperature and then centrifuged at 5000 g for 25 min. The supernatant was then used as carbon source in the liquid cultures [28].

2.5. Analytical methods

2.5.1. Dry cell weight

Samples were withdrawn from shake flasks at regular intervals and analyzed for biomass and PHB concentrations. Total dry biomass (bacteria and PHB) weight was evaluated gravimetrically by centrifuging the culture (after appropriate dilution) at 5000 g for 40 min, washing the sediment with distilled water, filtering via 0.2 µm millipore filter, and finally, drying at 80°C overnight. The additional mass of the pre-weighted filter was considered as the total biomass.

2.5.2. PHB Measurement

The amount of PHB was determined spectrophotometrically and chemical method was used to extract PHB [35-37]. The samples (10 mL) obtained from the shake flasks were centrifuged at 5000 g for 45 min. The solid pellets were re-suspended, washed with 1 ml equal portions of water, acetone, and ether, and centrifuged for 30 min at 5000 g. Subsequently, chloroform was added and allowed to boil in an ultrasonic bath at 100°C for 10 min and afterwards, incubated at 30°C for 24 h to evaporate chloroform. The obtained white powder was dissolved in concentrated H_2SO_4 (2.5 mL 96%) and heated at 100°C for 15 min. After cooling down to room temperature, the amount of PHB in the solution was determined photometrically at 235 nm against sulfuric acid blank.

A standard curve, correlating PHB concentration to the absorbed light intensities, was also generated by using pure PHB. A sample containing 5 to 50 µg PHB in chloroform was transferred to a clean test tube. When the chloroform was evaporated, 10 mL of the concentrated H_2SO_4 was added and heated in a water bath at 100°C for 10 min. After cooling down, its absorbance was measured at 235 nm against sulfuric acid blank. The standard curve was established with PHB concentrations ranging from 0 to 8 mgL^{-1} PHB.

2.6. Response Surface Methodology

RSM [30, 31] was used to find a statistical model to determine the optimum concentration of nutrient sources in culture media as independent variables (Table 1). A 2^n factorial Central Composite Design (CCD) was established and MINITAB software (Minitab® Inc. v17) was used to develop a model to optimize the concentration of the components. An experimental design of 32 experiments was formulated using the MINITAB software. All experiments were conducted in triplicate and the responses (i.e. mean values calculated for biomass and PHB concentrations as dependent variables) were imported into the software to optimize the substrate concentrations. Surface plots (3D) were generated to understand the interactions between molasses, urea

and CSL. Finally, the model was used to determine the optimal composition to simultaneously enhance biomass and PHB concentrations. To check validity of the predicted substrate concentrations, bacterial growth and PHB production were experimentally assessed in the optimized medium.

3. Results and discussion

3.1. Comparison of different carbon sources

The preliminary studies revealed that, in media with different carbon sources, the maximum PHB concentration could be achieved in 120 h. Therefore, under identical environmental conditions, each experiment was consistently carried out in triplicate for 120 h to make it possible to compare the media in terms of the obtained PHB concentrations. Figure 1a compares biomass and PHB concentrations obtained via four different selected carbon sources. According to the obtained results, after 120 h, maximum biomass concentration of 1.74 gL^{-1} and PHB content of 0.49 gL^{-1} were obtained in medium supplemented with cane molasses as the sole carbon source. Soya beans turned out to be the second best compound in terms of PHB production among the other carbon sources (Figure 1a). However, as compared to cane molasses, the PHB content was much lower. Minimum biomass and PHB were obtained with CSL. This may be due to the absence of the necessary enzymes required to hydrolyze the CSL carbon sources to simple carbohydrates, which can be easily utilized by *R.eutropha*. Therefore, with the highest PHB and biomass yields, cane molasses was used as carbon source in the rest of the experiments to optimize the culture medium for PHB production.

3.2. Effect of treating cane molasses

In order to precipitate and remove the heavy metals and suspended impurities, cane molasses was treated with different chemicals prior to being added to the culture medium. According to the obtained results (Figure 1b), highest bacterial growth (3.07 gL^{-1}) and PHB concentration were recorded when the molasses was pretreated with sulfuric acid.

This implies that the interfering substances which can hinder bacterial growth and PHB production can be effectively eliminated from the cane molasses by sulphuric acid treatment.

However, it should be noted that, according to Figure 1b, the ratio of intracellular PHB to biomass in treated and untreated molasses was not changed significantly (30% and 33%, respectively). This indicates that, although treating cane molasses with sulphuric acid could support and improve *R. eutropha* growth, it could not affect PHB synthesis and thus, the amount of PHB in each cell remained intact.

This can be explained, in part, by the biochemical characteristics of the culture medium composed of industrial compounds, which might not be able to properly stimulate PHB synthesis in *R.eutropha*.

In the published experimental studies, a wide range of PHB producing capabilities is reported. Such inconsistencies in PHB production can be explained by the microbial strain used to produce PHB, the composition of the substrates, the cultivation system, and in general, the conditions employed for microbial growth and PHB production. However, the results obtained in the present study are comparable with the published experimental results which have used the same strain to produce PHB [12, 25]. For example, Arun *et al.* (2006) reported a minor PHB production from untreated molasses in shake flask culture, whereas by using pure chemical substrates, Khanna and Srivastava (2005b) were able to achieve relatively higher concentrations of PHB and biomass.

3.3. Bacterial growth and PHB production patterns

Shake flask cultivation was performed with *R. eutropha* at 30°C and 150 rpm in the medium supplemented with cane molasses treated with sulphuric acid. Maximum biomass concentration of 3.1 gL^{-1} and PHB concentration of 1.05 gL^{-1} were recorded during the 120 h cultivation period. To better understand the bacterial growth and production patterns, samples were withdrawn at regular intervals and analyzed for biomass and PHB concentrations (Figure 2). An important feature observed in the growth curve of *R. eutropha* was a lag phase within the first 24 h. Adaptation to environmental and growth conditions would be the reason of such lag phase in bacterial growth. This means that, during the first 24 h adaptation period, *R. eutropha* may secrete essential enzymes to facilitate its proliferation by degrading the available nutrient sources.

3.4. Response Surface Methodology

RSM is a sequential exploratory approach to establish a relationship between multiple variables and a system response. In this analysis, in order to predict the responses, a model will be developed by fitting the obtained experimental data to a generalized smooth curve. By choosing the range of each variable, surface plots would be generated to delineate predicted responses over all the possible combinations of variables (i.e. design surface).

Table 1. Range of variables (i.e. concentration of media components) involved in the Central Composition Design to optimize the medium composition

Variables	Symbols	Level				
		-2	-1	0	1	2
Cane molasses (gL^{-1})	A	20	50	100	150	200
CSL (gL^{-1})	B	0.5	1.25	2	2.75	3.5
Urea (gL^{-1})	C	0	0.75	1.5	2.25	3
KH_2PO_4 (gL^{-1})	D	0.5	1	1.5	2	2.5
$MgSO_4.7H_2O$ (gL^{-1})	E	0.1	0.55	1	1.45	1.9

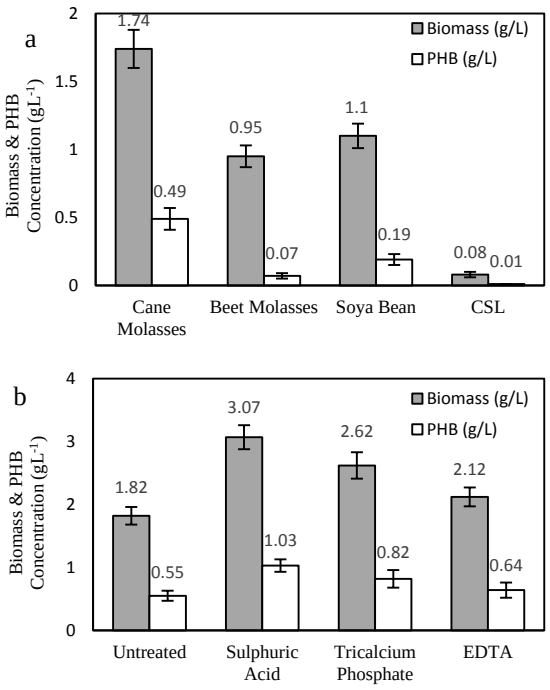

Figure 1. (a) The amount of biomass and PHB concentration in various carbon sources at 30°C and pH=7. Error bars represent standard deviations **(b)** The amount of biomass and PHB concentration obtained at 30°C and pH=7 in media composed of cane molasses as sole carbon source with various pretreatments. Error bars represent standard deviations.

Such surface plots can be used to visualize relations between the experimental levels of each variable and the corresponding responses. Optimum conditions can then be deduced from the surface plots. In this analysis, six replicates are required to estimate the error of sum of squares.
Therefore, a step-by-step approach of the response surface analysis can establish a robust relation between the variables and the observed responses, which would be more efficient than traditional factorial design [30].

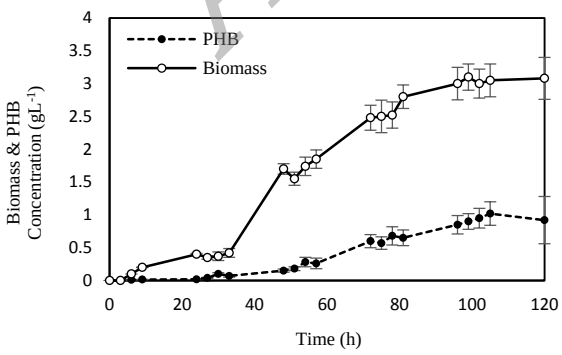

Figure 2. The amount of biomass and PHB concentration obtained in the course of experimental studies, indicating bacterial growth and PHB production patterns in the medium supplemented by pretreated cane molasses. Error bars represent standard deviations.

Here, the main culture medium components (Table 1) were considered as the five influencing factors (i.e. variables) to optimize the response (i.e. PHB production).

According to Table 1, each factor was tested at five different levels to verify whether lack or abundance of any component can promote PHB production in the culture medium.
The range of each factor was determined based on the central value (i.e. assigned concentration) and the sensitivity of the responses to that factor [30]. The CCD was then used to design the experiments to optimize the culture medium. A total of 32 experiments with different combinations of molasses (A), CSL (B), urea (C), KH_2PO_4 (D) and $MgSO_4.7H_2O$ (E) were designed by the software and conducted to obtain responses. Table 2 shows the distribution of the factors and the corresponding responses obtained experimentally. The experimental data were then fitted to the following second order polynomial equation:

$$Y_i=a_0+a_1A+a_2B+a_3C+a_4D+a_5E+a_{11}A^2+a_{22}B^2+ a_{33}C^2+a_{44}D^2+a_{55}E^2+a_{12}AB+a_{13}AC+a_{14}AD+ a_{15}AE+a_{23}BC+a_{24}BD+a_{25}BE+a_{34}CD+a_{35}CE+ a_{45}DE+\varepsilon \quad \text{Eq (1)}$$

where Y_i (i = 1 for biomass and 2 for PHB) is the predicted response, ε is the calculated error, a_0 is the value of the fitted response at the center point of the design, and a_i, a_{ii}, and a_{ij} are the linear, quadratic and cross point coefficients, respectively. These coefficients can be used to characterize the relation between the variables (concentration of nutrients) and the responses (i.e. biomass and PHB concentrations).

By importing the obtained responses (Table 2) into the software, coefficients of the second order polynomial (Equation 1) were computed (Table 3). Figure 3a shows the response surfaces plotted based on the calculated coefficients for cane molasses, CSL, and urea as the most important components. This information can be used to elucidate the interactions between such influencing factors. The surface plots revealed that, for high growth and PHB accumulation, the highest levels of molasses and CSL concentrations should be utilized (Figure 3a). Also, according to Figure 3a, the obtained plot for PHB production indicated that the urea concentration had negligible impact on PHB accumulation in this medium.

To evaluate the significance of each variable and their interactions, the obtained responses were also analyzed by analysis of variance (ANOVA). According to Table 4 and based on the calculated F-values, the first order and interaction terms were found to be more significant than the 2nd order terms. Also, the obtained values of 0.940 and 0.934 for the coefficients of determination (R^2) indicated that the models were in good agreement with experimental results.

Table 2. Central Composition Design matrix for bacterial growth and PHB production by *R. eutropha*.

Run No.	A	B	C	D	E	Biomass (gL^{-1})	PHB (gL^{-1})
1	-1	-1	-1	-1	-1	2.09±0.10	0.54±0.05
2	-1	1	-1	-1	-1	3.13±0.18	0.64±0.06
3	-1	-1	1	-1	-1	2.54±0.12	0.80±0.05
4	-1	1	1	-1	-1	3.21±0.08	0.17±0.03
5	-1	-1	-1	1	-1	2.44±0.21	0.71±0.05
6	-1	1	-1	1	-1	2.93±0.15	0.36±0.06
7	-1	-1	1	1	1	2.60±0.11	0.64±0.06
8	-1	1	1	1	1	3.12±0.11	0.35±0.04
9	1	-1	-1	-1	-1	1.94±0.08	0.60±0.08
10	1	1	-1	-1	1	4.72±0.22	1.34±0.16
11	1	-1	1	-1	1	2.94±0.19	0.88±0.8
12	1	1	1	-1	1	4.54±0.36	1.44±0.15
13	1	-1	-1	1	1	1.79±0.08	0.56±0.8
14	1	1	-1	1	-1	4.17±0.32	1.29±0.11
15	1	-1	1	1	-1	2.75±0.21	0.91±0.11
16	1	1	1	1	1	2.34±0.18	0.57±0.05
17	0	-2	0	0	0	1.06±0.07	0.47±0.07
18	0	2	0	0	0	3.19±0.23	0.79±0.09
19	0	0	-2	0	0	2.87±0.17	0.88±0.07
20	0	0	2	0	0	3.06±0.37	0.86±0.06
21	0	0	0	-2	0	3.04±0.25	0.91±0.06
22	0	0	0	2	0	2.59±0.18	0.78±0.08
23	-2	0	0	0	0	1.92±0.23	0.08±0.00
24	2	0	0	0	0	2.92±0.33	0.74±0.06
25	0	0	0	0	-2	3.51±0.28	1.07±0.11
26	0	0	0	0	2	3.02±0.19	0.78±0.08
27	0	0	0	0	0	2.73±0.36	0.77±0.08
28	0	0	0	0	0	2.64±0.20	0.77±0.08
29	0	0	0	0	0	2.85±0.28	0.84±0.09
30	0	0	0	0	0	2.90±0.33	0.79±0.05
31	0	0	0	0	0	2.81±0.19	0.83±0.07
32	0	0	0	0	0	2.90±0.24	0.78±0.07

Table 3. Coefficients of the fitted second order polynomials (Equation 1), characterizing the relationship between responses (i.e. biomass and PHB concentrations) and process variables (i.e. media composition)

Coefficient	Biomass (Y_1)	PHB (Y_2)
a_0	3.333	1.313
a_1	0.173	0.299
a_2	0.591	0.084
a_3	0.157	-0.021
a_4	-0.204	-0.107
a_5	-0.071	-0.017
a_{11}	-0.098	-0.166
a_{22}	-0.185	-0.076
a_{33}	0.066	0.022
a_{44}	0.020	0.012
a_{55}	0.155	0.045
a_{12}	0.039	0.178
a_{13}	-0.553	-0.078
a_{14}	0.117	0.049
a_{15}	0.183	-0.071
a_{23}	-0.283	-0.274
a_{24}	-0.356	-0.213
a_{25}	0.246	0.243
a_{34}	-0.008	-0.177
a_{35}	0.462	0.204
a_{45}	-0.539	-0.072

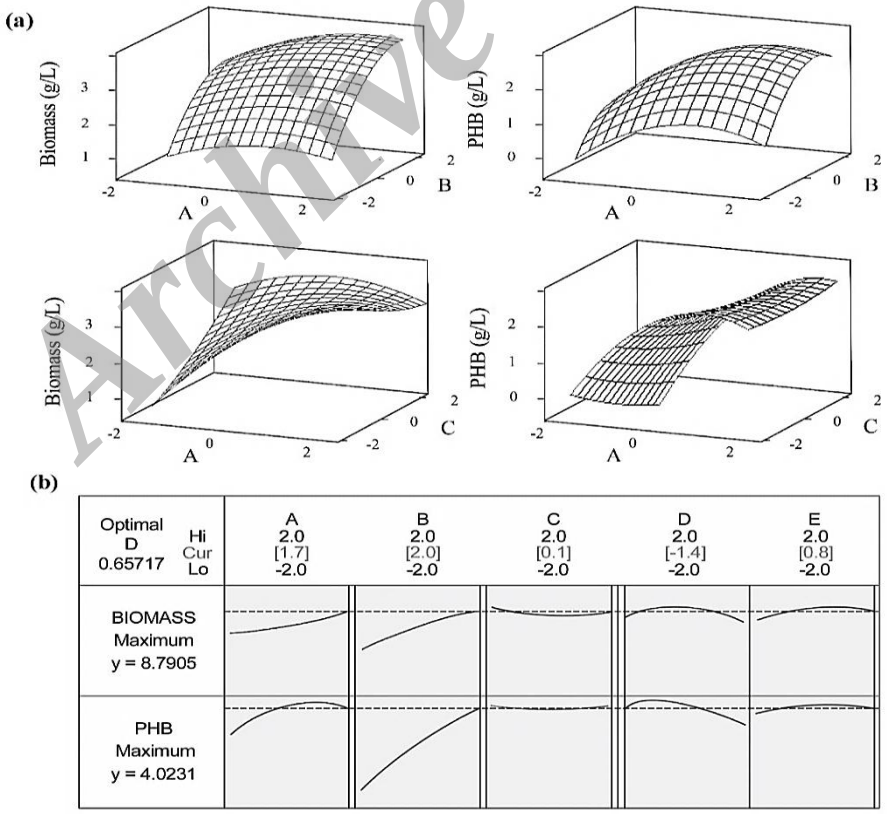

Figure 3. (a) Response surfaces showing simultaneous effect of cane molasses (A) and CSL (B) concentrations as well as cane molasses (A) and urea (C) concentrations on the obtained biomass and PHB concentrations. **(b)** Composition of the optimized medium, determined by Response Optimizer, to enhance PHB production.

Table 4. Analysis of variance for the fitted 2nd order polynomial models.

		Biomass (Y_1)				PHB (Y_2)			
Source	df	SS	MSS	F	P	SS	MSS	F	P
Regression	20	21.9956	1.0997	8.66	0.005	6.7001	0.3350	7.79	0.003
1st order terms	5	8.7876	1.7575	13.84	0.003	1.8494	0.3698	8.60	0.005
2nd order terms	5	2.3160	0.4632	3.66	0.034	1.0566	0.2113	4.91	0.013
Interaction	10	6.4150	0.6415	5.05	0.009	2.8261	0.2826	6.57	0.005
Residual error	11	1.3967	0.1269			0.4730	0.0430		
Pure error	5	0.0760	0.0152			0.0119	0.0023		
Total	31	42.3076				13.3781			
R^2			94.0%				93.4%		

3.5. Optimized medium

Facilitated by MINITAB Response Optimizer (a non-linear mathematical optimization procedure), the optimum concentrations to yield maximum biomass and PHB were determined by simultaneous optimization of the fitted biomass and PHB yield polynomials (Equation 1) (Figure 3b). Maximum biomass concentration of 8.79 gL^{-1} and PHB concentration of 4.02 gL^{-1} were predicted by the MINITAB Software. Shake flask studies were then carried out using the determined optimum concentrations (187 gL^{-1}, 3.50 gL^{-1}, 1.58 gL^{-1}, 0.80 gL^{-1}, and 1.36 gL^{-1} for cane molasses, CSL, urea, KH_2PO_4, and $MgSO_4.7H_2O$, respectively) to verify how PHB production would be altered in the optimal medium. Figure 4 shows the growth pattern of *R. eutropha* in the predicted optimal medium. A maximum of 5.03 gL^{-1} biomass and 1.62 gL^{-1} PHB was obtained by using the optimum concentrations after 120 hours, representing 57% and 40% of the predicted values for biomass and PHB production, respectively. However, it should be affirmed that, although the amounts of biomass and PHB were less than the predictions, they were the highest values achieved throughout the study.

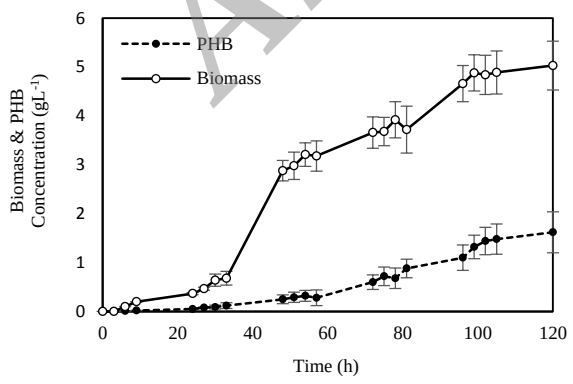

Figure 4. The amount of biomass and PHB concentration obtained in the course of experimental studies, indicating bacterial growth and PHB production patterns in the RSM optimized medium. Error bars represent standard deviations.

Additionally, in the optimized medium, the ratio of intracellular PHB to biomass was remained intact at 33%. This indicated that the PHB synthesis in *R. eutropha* could not be affected by the medium composition and the increased amount of PHB was just due to the higher bacterial growth and enhanced biomass accumulation in the optimized medium. Accordingly, it could be concluded that the present culture media were not able to stimulate PHB synthesis by *R. eutropha* and thereby, the peak PHB production could not be achieved. However, the main objective of the present study was evaluating how low cost and renewable components can be utilized to allow PHB production, and then to carry out media optimization studies to determine the maximum possible PHB production in such media. Therefore, more investigations should be conducted to determine which modifications are required to stimulate PHB synthesis in the medium composed of cane molasses as sole carbon source.

4. Conclusion

In order to reduce the cost of PHB production, instead of expensive pure chemicals, in this work, domestic low cost and renewable resources including beet molasses, cane molasses, soya bean, and corn steep liquor were used as carbon sources to produce PHB by *R. eutropha*. Also, urea and corn steep liquor were used as nitrogen, vitamin, and mineral sources. Preliminary investigations revealed that the highest biomass and PHB concentrations could be achieved by using cane molasses. Therefore, to improve bacterial growth and PHB production, different methods were used to treat cane molasses prior to being added to the culture media. Based on the obtained results, sulfuric acid treatment was selected as the most efficient method in removing the interrupting heavy metals and suspended impurities of the cane molasses. Subsequently, RSM was used to optimize the medium composition to enhance PHB production. Using the optimal medium, 58% and 41% improvements were observed in biomass and PHB concentrations, respectively. However, when

compared to the previous experiments, the results obtained in the optimal medium indicated that the new composition was not able to stimulate PHB synthesis in *R.eutropha*, meaning that the enhanced PHB concentration was just related to the higher number of cells in the optimal medium. Therefore, although the results of the present study may inspire industrial-scale biotransformation of renewable low cost resources, more investigations are required to assess how PHB synthesis can be stimulated in *R.eutropha* when growing in the medium composed of cane molasses as the sole carbon source.

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6. Conflict of interest

The authors report no conflict of interests relevant to the subject of the present manuscript.

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