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Original Article

Scaling up, Kinetic modeling, and Economic analysis of poly (3-hydroxybutyrate) production by *Bacillus cereus* isolate CCASU-P83

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ABSTRACT

Polyhydroxyalkanoates (PHA) are environmentally friendly polymers produced by many bacteria under nutrient-limited conditions. However, their commercialization is hindered by production expenses. The present study aimed at cost-effective and efficient production of poly(3-hydroxybutyrate) (PHB) by *Bacillus (B.) cereus* isolate CCASU-P83. Through one factor at time optimization study on shake flask, *B. cereus* CCASU-P83 produced about 50 % polymer per dry weight after 48 hours incubation time. For better evaluation of the fermentation process, kinetic modeling using the Logistic and Leudking-piret models was applied. A preliminary economic analysis was carried out and leads to a 30.8 % reduction in the total cost. In comparison to the findings obtained on the level of shake flask, scaling up to the bioreactor resulted in producing about 53% PHB per dry weight after only 24 hours incubation. These models concluded that *B. cereus* produced PHB during the growth phase. Analysis of molecular weight of the produced polymer displayed a 26900 g/mole molecular weight with a polydispersity index (PDI) of 1.1. In conclusion, *B. cereus* CCASU-P83 is a potential candidate for industrial production of PHB polymer using corn oil in a short incubation period which highly reduced the cost of the production process.

Keywords: *Polyhydroxyalkanoates; Logistic; Leudking; Fermentation; optimization*.

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1. INTRODUCTION

A lot of attention is directed towards increasing the productivity of poly (3-hydroxybutyrate) (PHB), a plastic alternative, by different bacteria due to its high industrial potential. This polymer has similar properties to synthetic polymers besides biodegradability and biocompatibility which facilitate its utilization in biomedical implantation and controlled release preparations. However, the high production cost of PHB polymer is an obstacle in the road of commercial use of this polymer **[1]**. Accordingly, a lot of trials were carried out to decrease this high cost by selecting the suitable microorganism, optimization of small and large scale production, kinetic modeling, and characterization of the produced polymer **[2]**. Both Gram-positive and Gram-negative bacteria from various genera are identified to produce polyhydroxyalkanoates (PHA) polymers. Physiologically, these myriads of bacteria differ

in the timing of PHA production. Some of them synthesize the polymer in the late log phase of the growth cycle and others in the stationary phase. However, all of them use it as a carbon source in starvation periods **[3]**. There are some bacterial species capable of producing the PHA polymers in an enormous amount suitable for industrial use for example Alcaligenes latus, Cupriavidus necator, and Pseudomonas putida **[4, 5]**. In addition to all of the previous examples, Bacillus species occupy a high rank in the industrial production of PHA biopolymer. They lack lipopolysaccharides which remove the burden of cumbersome product purification **[6]**. They have a high growth rate and excellent genetic stability **[3]**. The major drawback of using Bacillus species in PHA production is its sporulation which leads to PHA deposition **[7]**. Another drawback is the high cost of the raw materials used by Bacillus species which negatively impacts the applicability of large-scale production of PHA **[8]**. Thus, the development of an efficient submerged fermentation of Bacillus species using low-cost raw materials is crucial.

In our previous study, a lot of experiments were implemented to search for promising bacterial species capable of producing PHB polymer at an industrial scale. *B. cereus* isolate CCASU-P83 was selected owing to producing 13% PHB per dry weight after 48 hours of incubation using basal medium **[9]**. Therefore, this study aimed at investigating different environmental factors and culture conditions affecting PHB production by the respective isolate first on the shake flask followed by scaling up using a 14L bioreactor. Moreover, kinetic modeling and economic analysis were carried out to have new insights on the PHB production and its potential commercialization.

2. MATERIALS AND METHODS

2.1. PHB production on shake flask

B. cereus isolate CCASU-P83 (NCBI nucleotide accession code KC876035) is a soil bacterium capable of producing PHB polymer **[9]**. This isolate was subcultured on LB agar, incubated at 37 °C and maintained as a 20% (vol/vol) glycerol stock preparation and deposited in Culture collection Ain Shams University (CCASU) which is listed in the World Data Centre for Microorganisms (WDCM) (http://www.wfcc.info/ccinfo/collection/by_id/1186). For PHB production, the preparation of the pre-culture was carried out by culturing the bacteria into LB broth at 37 °C, 160 rpm, and 20 hours incubation. Then, the culture was carried out in the mineral salt medium (MSM) as shown in Table 1 where the inoculum size, shaking speed, temperature, and time was 5% v/v, 200 rpm, 37 °C, and 48 h, respectively.

2.2. Different conditions affecting the PHB production

Different aeration levels were tested by changing the ratio of culture medium to the Erlenmeyer flask headspace (90, 80, and 60% aeration). Moreover, variable initial pH (4, 5, 7, 8, and 9), inoculum sizes into MSM medium (0.5, 2, 5, and 10% v/v), and incubation temperatures (28, 37, and 40 °C) were investigated.

2.3. Effect of various media ingredients

2.3.1. The carbon sources

Various carbon sources were evaluated instead of glucose in the basal medium at the same concentration (0.7% w/v). The tested categories of carbon sources were monosaccharides, disaccharides sugars, sugar alcohol, polysaccharides, oils, and unrefined carbon sources such as Malt extract. Thereafter, the carbon source(s) gave maximum PHB production was tested at different concentrations (0.4%, 0.7%, 1.5%, 2% and 4% w/v) to select the optimum concentration.

2.3.2. The nitrogen sources

Two different groups of nitrogen sources were tested organic and inorganic compounds. Different concentrations of the optimum nitrogen source were investigated.

2.3.3. The multivalent minerals

Five sets of MSM flasks were used: One flask is an MSM medium devoid of all minerals and the other four flasks contain individual minerals in the MSM medium.

2.4. PHB production using the new medium

The new medium (coded 83M, **Table 1**) was created based on the optimum results for every tested factor or condition as previously reported **[10]**.

Table 1. Composition of MSM and newly formulated medium 83M used in PHB production

Name of ingredient	Basal medium (MSM)	Newly formulated medium (83 M)
Carbon source (amount/L)	Glucose (7 g)	Corn oil (7 mL)
Nitrogen source (amount/L)	Ammonium chloride (0.1 g)	Ammonium chloride (0.2 g)
Minerals (amount/L)	$MgSO_4 \cdot 7H_2O$ (0.2 g) $CaCl_2$ (0.01 g), Ferrous ammonium sulphate (0.06 g), trace elements solution* (1 mL)	No Minerals
ommon ingredients (amount/L)	$Na_2HPO_4 \cdot 12H_2O$ (10.2 g), KH_2PO_4 (1.5 g), NaCl (10 g)	

* -Trace elements solution contains (amount/L) ($CoCl_2$. 6 H_2O (0.2 g), H_3B0_3 (0.3 g), $ZnSO_4$. $7H_2O$ (0.1 g), $MnCl_2$.$4H_2O$ (30 mg), $NiCl_2$ (10 mg), $CuSO_4$. $5H_2O$ (10 mg).

- MSM and 83M media were sterilized by autoclaving. Glucose and trace elements solution were filter sterilized and were aseptically added to the autoclaved media with the indicated concentration.

2.5. Economic analysis

To preliminary evaluate the change in cost after applying the new medium on shake flask, an economic analysis **[11]** was carried out by calculating these parameters:

Change in total cost= Total cost of a new medium (P2)-Total cost in basal medium (P1) **[11]**.

2.6. Scaling up

PHB production was carried out in a 14 L Bioflo 310 glass bioreactor (New Brunswick Scientific, Edison, NJ, USA. The special conditions for the bioreactor were 1 vvm aeration (4 SLPM), 100% oxygen saturation, and uncontrolled pH. Different samples were collected for measuring the PHB concentration, biomass, PHB percentage per dry weight, and corn oil utilization **[12]**.To study the effect of aeration, other fermentation runs were carried out using the same conditions listed above except at different aeration rates of 0.5, 2, and 4 vvm.

2.7. Kinetic modeling

The fermentation process was described using two kinetics models; one describing cell growth and the other describing product formation. This was applied to the best fermentation run.

2.7.1. Cell growth kinetic model

A substrate-independent model described by logistic equation **[13]** was formulated as follows:

$$X = \frac{x_\circ \exp(\mu_m t)}{1-\left(\frac{x_\circ}{x_m}\right)(1-\exp(\mu_m t)} \quad \textbf{[13]}$$

where μ is the specific growth rate, μm is the maximum specific growth rate (h-1), x is biomass concentration concerning time (g/L) and XM is maximum cell dry weight concentration (g/L).

2.7.2. Product formation kinetic model

The biosynthesis of PHB can be represented by Luedeking–Piret type model **[14]**. The product formation rate is described as:

$$P_t = P_\circ + \alpha\left[\frac{X_\circ\, e^{\mu_m t}}{(1-(\frac{X_\circ}{X_m})(1-e^{\mu_m t})} - X_\circ\right] + \beta\left(\frac{X_m}{\mu_m}\right)\ln\left[1-{}^{X_\circ}/_{X_m}\ (1-e^{\mu_m t})\right]$$

[14]

Where α is growth-associated constant, β is non-growth-associated constant, x is biomass concentration and dx/dt is the rate of growth.

Kinetic model parameters were estimated by applying experimental data to the proposed models using software Graph Pad Prism version 5 by using nonlinear least-squares curve fitting with 95% confidence interval.

2.8. Molecular weight measurement

Molecular weight data were obtained by gel permeation chromatography (GPC) with a refractive index detector in the National research center, Dokki, Egypt. The extracted polymer was dissolved in dimethylformamide, filtered using a 0.45 μm syringe filter, and sent for measurement. A report is provided for each sample stating three important values: the number average molecular weight (M N), the weight average molecular weight (M w), and the polydispersity index (PDI).

2.9. Analytical Methods

2.9.1. Biomass determination

Biomass was expressed as dry cell weight which was derived from a calibration curve created between optical density (OD640 nm) and dry cell weight of the isolate CCASU-P83 **[10]**.

2.9.2. PHB concentration determination

It was determined using the spectrophotometric method **[15]**. The extracted PHB polymer was converted to crotonic acid by concentrated sulphuric acid and was measured at 235 nm.

3. RESULTS AND DISCUSSION

3.1. Shake flask production

3.1.1. Aeration and incubation temperature effect on PHB production

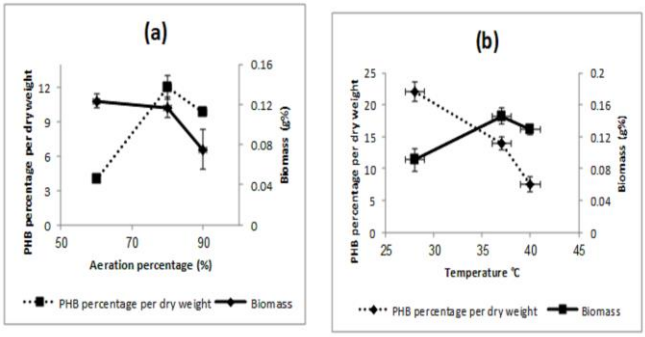

Fig 1. Effect of aeration **(a)** and incubation temperature **(b)** on PHB percentage per dry weight and biomass in B. cereus isolate P83

B. cereus isolate CCASU-P83 achieved its highest PHB percentage per dry weight using 80% aeration and at 28 °C **(Fig. 1)**. For the aeration level, these results coincide with many studies supporting that oxygen is a very important trigger for PHB production **[16, 17]**. Since the optimum incubation temperatures range

for PHB production is from 25-35 °C **[18, 19]**, thus our results fall in the correct zone.

3.1.2. Effect of pH and inoculum size

Results of testing different pH and inoculum sizes revealed that the highest PHB percentage per dry weight was established using pH 4 and 0.5% inoculum size. However, they were not selected as optimum conditions in further studies because this upheaval is due to a reduction in biomass rather than an increase in PHB accumulation. Thus, pH 7 and 5% inoculum size were used as optimum conditions because the organism accumulated the highest PHB polymer under these conditions.

3.1.3. Effect of different carbon and nitrogen sources on PHB production

Effect of different carbon sources on PHB productivity of the test isolate **(Table 2)** showed that the highest PHB percentage per dry weight was achieved using corn oil and paraffin oil. A lot of studies advocated the use of oil as an optimum carbon source **[20, 21]**. Borah and his coworkers (2002) mentioned that carbon sources either target biomass formation or PHB production **[16]**. This was brightly observed in the present study where glycerol, sucrose, maltose, and fructose enhanced the biomass formation only. Since paraffin oil and corn oil gave the best results so they were tested at different concentrations. It was observed that the best concentration for both oils was 1.5% v/v based on the high PHB percentage per dry weight previously determined **[10]**. Consequently, corn oil (0.7%) was chosen as the optimum carbon source.

Table 2. Effect of replacement of glucose with other carbon sources on PHB percentage per dry weight and biomass of *B. cereus* isolate CCASU-P83

Carbon source (0.7 %)	PHB percentage per dry weight	Biomass (g%)
Glucose (control)	13	0.1
Galactose	10	0.0936
Fructose	6	0.126
Maltose	2	0.1465
Lactose	20	0.0233
Sucrose	2.5	0.1728
Mannitol	16	0.0611
Glycerol	4.4	0.1928
Starch	14	0.0848
Paraffin oil	27	0.0414
Corn oil	24	0.051

In the present study, the limitation of nitrogen was chosen to trigger PHB production because ammonia is considered a pivotal factor for the uncoupling of growth and PHB **[22]**. The results of the effect of different nitrogen sources on PHB percentage per dry weight and biomass formation are displayed in **Table 3**. It was found that both ammonium chloride and peptone produced a

comparable high PHB percentage per dry weight. Trakunjae *et al* (2021) tested both inorganic and organic nitrogen sources for optimum production of PHB by *Rhodococcus* and they reported that the inorganic nitrogen source, potassium nitrate, gave the best results **[23]**. On the other hand, Tripathi *et al* (2012) advocated the use of urea for optimum production of PHB by *Pseudomonas aeruginos* **[15]**. After testing different concentrations of both ammonium chloride and peptone, about 0.1 g/L of ammonium chloride attained the highest PHB percentage per dry weight.

Table 3. Effect of replacement of ammonium chloride with different nitrogen sources on PHB productivity per dry weight and biomass of *B. cereus* isolateCCASU-P83

Nitrogen source (0.1 g/L)	PHB percentage per dry weight	Biomass (g%)
Ammonium chloride (control)	13	0.1165
Yeast extract	11	0.0504
Peptone	12.8	0.0983
Beef extract	2.2	0.1218
Urea	3.6	0.1195
Tryptone	9	0.0442
Potassium nitrate	2	0.0482
Ammonium nitrate	5	0.2141

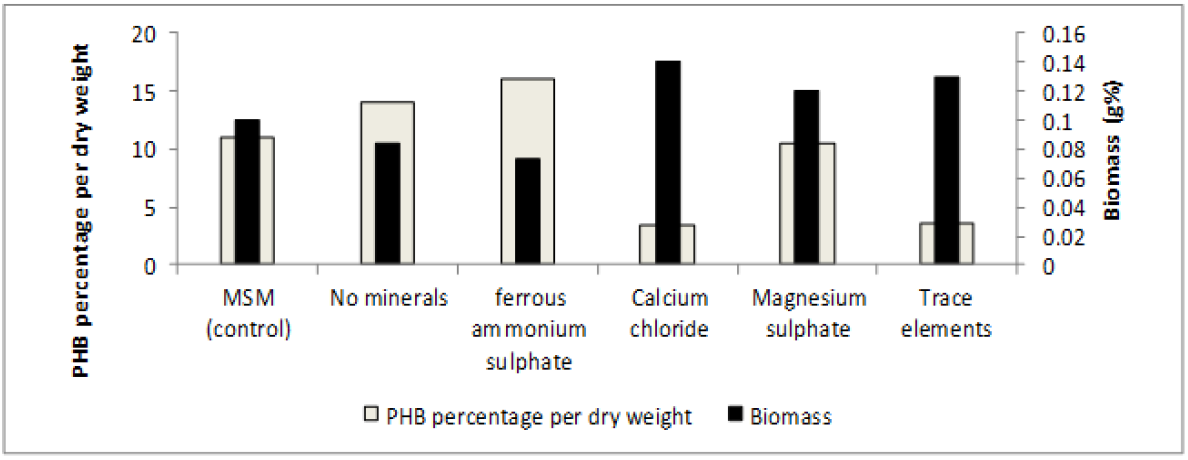

Fig 2. Effect of minerals on PHB percentage per dry weight and biomass formation of *B. cereus* isolate P83

3.1.4. Effect of minerals on PHB production

As shown in **Fig. 2**, the maximum PHB percentage per dry weight was achieved using MSM devoid of all minerals and that containing ferrous ammonium sulfate only. However ferrous ammonium sulfate decreased biomass in

comparison to MSM devoid of minerals. Thus, MSM medium devoid of minerals were used for further studies. This agreed with what Sangkharak and Poonsuk (2008) noticed that both calcium chloride and magnesium sulfate have a negligible effect on PHB production **[22]**.

3.1.5. Time course of PHB production by *B. cereus* using newly formulated medium (83 M)

After gathering all the optimum factors in the new medium (83 M), the results were fascinating. *B. cereus* isolate CCASU-P83 showed a pronounced increase in PHB percentage per dry weight from 13% to 50% **(Table 4)**. This implies that the maximum PHB production percentage per dry weight was about 4 fold higher in the new medium (83 M) compared to basal medium after equal incubation periods of 48 h. This was higher with what reported by Valaprill *et al* (2007) 38% PHA production for *B.cereus* using glucose **[24]**. On the other side, Sangkharak and Prasertsan (2012) observed 64% PHA production using *B. cereus* using Palm oil effluent after 96 h of incubation **[25]**. However, our results are better in terms of a short incubation period (48 h) rather than (96 h) which is more cost-effective. Furthermore, Chaudhry *et al* (2011) used corn oil as a feeding substrate for different *Pseudomonas* species for PHA production and about 35% per dry weight was the best achievement **[26]**.

Table 4. The maximum productivity and PHB production percentage per dry weight of *B. cereus* isolate in basal and newly formulated media

Isolate	Medium applied	Maximum PHB production percentage per dry weight (in hours)
B. cereus CCASU-P83	Basal medium (MSM)	13 % (48 h)
	83M	50 % (48 h)

3.2. Economic analysis

By applying a simple economic estimation of the total production cost of both the basal medium and the new media, about a 30.8% reduction in total cost was calculated as shown in **Table 5** after using the new medium. This is an incredible reduction in the cost of PHB production by this isolate which predisposes to a cost-effective process. The reason behind this reduction is the usage of cheap carbon sources such as corn oil instead of glucose. The cost of carbon source contributes to 28% of the total cost of PHA production so removing this obstacle paves the way for proper commercialization **[27]**. Naranjo and his coworkers (2013) stated also a reduction in the price of PHB production upon replacing glucose with glycerol **[6]**.

3.3. Scaling up

The use of the shake flask conditions (28 °C temps, 200 rpm agitation, 1 vvm aeration) in 14 L laboratory bioreactor led to a 1.4-fold increase in PHB production in a shorter incubation time than that of shake flask (24 rather than 48 h) as shown in **Fig. 3**. Batch fermentation is known to enhance the production of PHB polymer **[27]**. Further studies were carried out for the respective isolate to study the effect of aeration on PHB production. It was found that upon decreasing aeration to 0.5 vvm, the PHB production and biomass decreased significantly. Peña and his coworkers (2014) pointed that decreasing

aeration decreases the volumetric PHB production which agrees with our results **[28]**. On the other hand, increasing the aeration level to 2 vvm **(Fig. 4)** led to an increase in PHB production with a slight decrease in biomass than that obtained using 1 vvm aeration. Accordingly, increasing the aeration level from 1 vvm to 2 vvm was associated with an increase in the PHB percentage per dry weight from 49% to 53%. However, a further increase in aeration level to 4 vvm was associated with a decrease in PHB production and biomass. From the obtained results, the optimum aeration level for PHB production by *B. cereus* isolate CCASU-P83 was 2 vvm which was used for further studies. In a nutshell, *B. cereus* isolate CCASU-P83 produced 0.266 g/L with a polymer content of 53% per dry weight which was higher than that produced by different *Bacillus* species in other studies **[29-31]**.

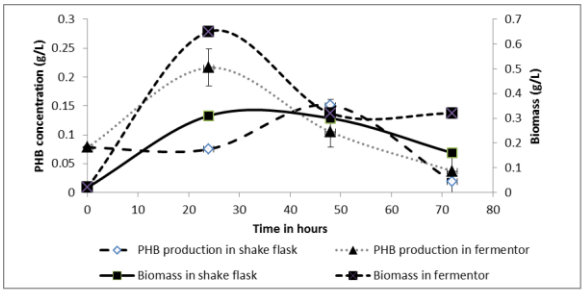

Fig 3. PHB production and biomass formation profiles of *B. cereus* isolate P83 in the shake flask and in the bioreactor using the same production media and culture conditions

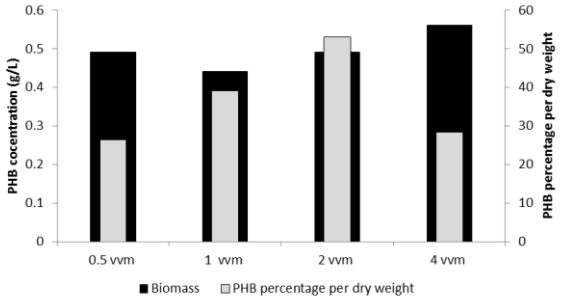

Fig 4. Maximum PHB percentage per dry weight by *B. cereus* isolate P83 in the laboratory bioreactor at different aeration rates after 24 h of fermentation. Conditions applied: uncontrolled initial pH of 7.2; temperature of 28 °C; agitation rate of 200 rpm; and inoculum size 5% v/v

3.4. Kinetic modeling

The ability of *Bacillus cereus* isolate to produce PHB polymer in 24 h intrigued us to search whether the production of PHB was during the growth phase or stationary phase. Thus we used mathematical modeling which is mainly used to describe the microbial performance during the fermentation process by mathematical equations **[32]**. Two mathematical models, Logistic and Leudking piret, were used to describe microbial growth and product formation kinetics.

Logistic model mostly used to describe the bacteria growth kinetics during polymer production **[14]**. This model implies a directly proportional relationship between the rate of cellular growth and cell mass concentration. According to the obtained results of kinetic modeling, the used logistic model **(Equation 1)** fitted well to experimental culture conditions producing a good predictive model for microbial growth of the tested isolate with an r^2 value of approximately 0.99 **(Fig. 5A)**. The values of maximum specific growth rate and maximum biomass are shown in **Table 5**.

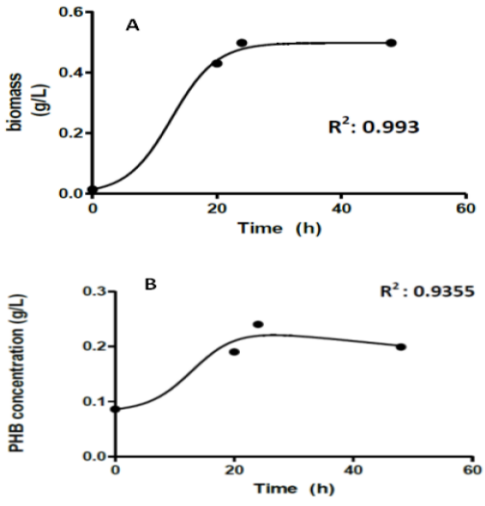

Fig 5. A) Cell growth kinetic model curves of fermentation for *B. cereus* isolate P83 in comparison to experimental data. **B)** Product synthesis kinetic model curves of fermentation for *B. cereus* isolate P83 in comparison to

experimental data. The dots represent the experimental results and the lines represent the data determined by the described kinetic model

The Luedeking-Piret equation was applied in the present study to describe the microbial behavior for polymer production. The Luedeking-Piret model categorizes the relationship between the product and the microorganism growth into Class 1 where microorganism growth is directly proportional to product formation where $\alpha>0$, $\beta= 0$; Class 2 where the partial relationship is found between the two systems so $\alpha >0$, $\beta>0$; and Class 3 where no relationship between the product and the microorganism growth so $\alpha= 0$, $\beta>0$ **[33]**.

In the present study, the obtained results revealed that the Luedeking-Piret model fitted very well to the experimental data **(Fig. 5B)**, and the growth associated constant (α) was higher than the non-growth associated constant (β) in this isolate, so the polymer is growth associated product; class 1. Growth-associated production means that the rate of polymer production parallels the bacterial growth rate. Many studies confirmed the growth associated nature of PHB production where Khan *et al.* (2013) reported this phenomenon in *Cupriavidus necator* H16 **[34]**. Moreover, Yang *et al* (2006) mentioned that Zoogloea sp. GY3 produces PHA during growth **[25]**.

3.5. Molecular weight measurement

Molecular weight is a fundamental property of PHA polymer. There is a direct relationship between the polymer's mechanical strength and its molecular weight **[35]**. The values of the molecular weight measured are listed in **Table 6**. Cuellar *et al*. (2011) reported that PHB average molecular weight ranges from 10 4 to 10 6 g/mole **[36]**. Another important property of a polymer is its polydispersity index (Mw/Mn). In this study, low polydispersity was recovered by *B. cereus* isolate CCASU-P83 (1.1) which supports the usage of PHB polymer recovered in biomedical applications.

Table 5. Parameter values calculated using Logistic and Luedeking–Piret equation

Parameter	Value
Logistic model (Equation 1)	
X° (g/L)a	0.014
Xm (g/L)b	0.498
µm (h-1)c	0.2781
Luedeking–Piret model (Equation2)	
α (Growth associated constant)	0.3168
β (Non-growth associated constant)	0.002197

a X°: initial biomass, b X m : Maximum biomass, c µm : Maximum specific growth rate

Table 6. The molecular weight of PHB synthesized by B. cereus isolate CCASU-P83

Isolate	MW	Mn	PDI	NP
B. cereus isolate CCASU-P83	26900 g/mole	24000 g/mole	1.1	3.3*1018

MW, weight average molecular weight; Mn, number average molecular weight; PDI, polydisperisity index; Np, no. of polymer chains per liter

Conclusion

The soil bacterium *B. cereus* CCASU-P83 produces PHB polymer using corn oil and ammonium chloride as carbon and nitrogen sources, respectively. Under optimum conditions obtained through experimentation, this strain can accumulate 50% PHB per dry cell weight. Using the optimized medium there was an improvement both technically and economically. Technically the optimized medium improved the production from 13% to 50% per dry cell weight and economically there was a 30.8% reduction in total cost. Furthermore, a higher PHB percentage was attained using a 14 L bioreactor in a shorter incubation period. Finally, the mathematical modeling of both growth and polymer production concluded the growth associated production of PHB polymer using *B. cereus* isolate CCASU-P83.

Declarations

Ethics approval and consent to participate

Not applicable

Data availability

Data supporting the conclusion of the study is available in the manuscript and the nucleotide DNA sequencing of the 16S ribosomal RNA is available under the NCBI GenBank accession code, KC876035.

Conflict of interest

The authors declare that they have no competing interests

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Author contributions

All authors contributed extensively to the work presented in this paper. NS designed, performed the experiments, and wrote the manuscript. KM, MA, and MM designed the experiments, analyzed data, and revised the manuscript. NH supervised and revised the manuscript. All authors discussed the results and commented on the manuscript at all stages

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Consent to publish

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article in the main manuscript.

Competing interests

No competing interests were declared by the authors.

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