Kinetic model for polyhydroxybutyrate production by *Bacillus aryabhattai* MH997667.1 in a batch culture using different agitation speeds

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Background
Slow degradation of synthetic plastics represents a high environmental hazard; therefore, there is an essential requirement to substitute them with eco-friendly products. Polyhydroxybutyrate (PHB) is a biodegradable biopolymer and also has several industrial, agricultural, and medical applications. Scaling up the production of PHB is still a problem due to the numerous parameters tangled in the fermentation processes.

Objective
The present work seeks to scale up polyhydroxybutyrate production by *Bacillus aryabhattai* MH997667.1 from shaken flasks to a 5L-bench top bioreactor with previously optimized media by applying a batch fermentation strategy.

Materials and methods
Different agitation speeds (200, 250 and 300 rpm) were tested in a 5L-bench-top bioreactor with a working volume of 3L. A kinetic model (logistic and Luedeking–Piret) that describes the microbial biomass and polyhydroxybutyrate (PHB) production is used to expect the performance of batch fermentation of *Bacillus aryabhattai* MH997667.1.

Results and conclusion
The optimum PHB yield (2.32 g/l) was expressed at 250 rpm agitation after 28 hours of fermentation. The experimental data were also fitted with the logistic and Luedeking–Piret equations for growth and PHB formation, respectively. The mathematical model proposed for batch fermentation revealed that the simulated data showed a good fit with the experimental results obtained during the first 24 h of PHB production at 250 rpm, where the productivity was 0.095 g $L^{-1}h^{-1}$. Our data suggest that agitation speed had a significant effect on PHB production and the 250 rpm agitation speed is the optimum speed for PHB production using *Bacillus aryabhattai* MH997667.1 in batch fermentation.

Keywords:
Bacillus aryabhattai, kinetic model, logistic, Luedeking–Piret model, polyhydroxybutyrate, simulation

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Introduction

Polyhyroxybutyrate (PHB) is a biodegradable, thermoplastic, and biocompatible polyester accumulated intracellularly by many microorganisms in the presence of excess carbon and limited nutrients such as nitrogen or phosphorous [1–3]. PHB is produced by many bacterial strains such as *Ralstonia eutropha*, *Azotobacter* sp., *Bacillus* sp., and *Pseudomonas* sp.; it can also be isolated from different environments such as activated sludge, rhizosphere, oil-contaminated soils, and hypersaline one [4–10].

PHB has many industrial applications such as in packaging, cosmetic containers, agriculture, paint industry, pharmacy, and food industry [3]. The commercialization of PHB is still limited due to its high cost of production compared with petroleum-derived plastics [11]. Several previous researches have reported the utilization of economic substrates such as methanol, ethanol, starch, whey, and beet molasses to decrease the cost of PHB production [12–16], wheat hydrolysate, fungal extract [17], soy cake [18], and cane-molasses [19]. It was also reported that the fermentation process and the product recovery contribute to the major cost [20]. To reduce the cost by optimizing the fermentation process, the operon

responsible for PHB production was expressed in other microorganisms such as *Escherichia coli* [21,22].

The production of PHB is a complex process that depends mainly on fermentation parameters, phase of PHB production, strain, metabolic pathway, carbon source, and other nutrient depletion [23]. A mathematical model useful for the production of PHB helps in the design and maximization of the process. The kinetic model suitable for PHB production should include balances on cell mass, substrate utilization, product concentration, and a single limiting substrate [24].

The present research aimed to investigate the effect of various agitation speeds (200, 250, 300 rpm) on PHB production in batch culture; furthermore, the kinetic parameters for the cell growth were determined.

Materials and methods

Microorganism

The bacterial strain used in the present study was previously isolated from Egyptian soil in El-Giza Governorate and was previously identified as *Bacillus aryabhattai* MH997667.1 using 16 S rDNA [25].

Batch culture study

Bacterial inoculum 10% (v/v) of 24 h freshly prepared culture, incubated at 30°C for 48 h at 200 rpm on a rotary shaker was used to inoculate 3 L of culture medium contained in the bioreactor. The production of PHB by *B. aryabhattai* was carried out by fermentation in a bioreactor using a batch strategy. Fermentations were performed in a 5 L stirred-tank bioreactor (B. Braun Biotech International, MCU-200).

The fermentation medium (MSM) [15] composed of (g/l) beet molasses 30, KH_2PO_4 1.5, Na_2HPO_4 3.525, $MgSO_4.7H_2O$ 0.2, $CaCl_2$ 0.02, NH_4Cl 0.75, ferric citrate 0.0015, and the pH was adjusted at 8.0. The bioreactor and the fermentation medium were previously sterilized by autoclaving at 121°C for 20 min. Cultivation was conducted at 30°C± aeration rate (1 v/v/m) and dissolved oxygen DO were measured using an autoclavable sensor (Mettler-Toledo, Switzerland). The effect of agitation speed on PHB production was studied at 200, 250, and 300 rpm. Samples were removed from the bioreactor for analysis. Both pH and OD were recorded throughout the batch process. Tween 80 antifoam was added to avoid the formation of foam.

Analytical methods

Dry cell weight estimation

Cell samples from the fermenter were harvested by centrifugation for 15 min at 10,000 rpm and at 4°C. The supernatant was discarded and the pellets were washed twice with water and dried at 80°C until constant weight [26].

Biopolymer extraction and quantification

Extraction of the biopolymer was according to the method developed by Law and Slepecky [27]. For biopolymer quantification, the pellets were washed with acetone and ethanol and were suspended in 4% sodium hypochlorite and then incubated at 37°C for 1 h to remove the cell wall. The mixture was then centrifuged again at 10,000 rpm for 10 min, and the collected pellets were washed by adding acetone, ethanol, and water to remove cell lipids and other molecules with the exception of PHB granules. PHB granules were dissolved in hot chloroform and filtered through Whatmann no.1. To the filtrate, 10 ml of 98% H_2SO_4 was added and heated at 100°C for 10 min in a water bath. PHB was determined quantitatively as crotonic acid by measuring the absorbance at 235 nm in a UV spectrophotometer using H_2SO_4 solution as a blank. The amount of PHB (g/l) was determined by comparing absorbance readings with a standard crotonic acid curve [28].

Biopolymer analysis

Nuclear magnetic resonance (NMR) analysis was performed previously [25].

Manipulation of experimental data and mathematical modeling

Experimental results were fitted into curves with the highest R^2 to obtain a logical pattern of the process and eliminate experimental error. The curves fitted to the experimental results have polynomial equations of second order or higher, according to the results. These equations were differentiated with respect to the time to calculate the rate, either growth rate or production rate. The biomass was calculated from the equation of the curve, then the specific growth rate or specific production rate was calculated by dividing the rate over the biomass.

Logistic equation for microbial growth and Luedeking–Piret kinetic equation for product formation were proposed to describe the growth of *B. aryabhattai* MH997667.1 and production of PHB.

Logistic equation [29]

This equation correlates the growth rate (dX/dt) with maximum cell concentration (X_m) as follows:

$$\frac{dX}{dt} = \mu_m X\left(1 - \frac{X}{X_m}\right)$$

where

X=cell concentration, g/l

t=time, h

μ_m = maximum specific growth rate, h^{-1}

X_m = maximum cell concentration, g/l.

The integrated form of Eq. (1) using $X=X_0$ (t=0) gives the following expression (Eq. (2):

$$X = \frac{X_0 exp(\mu_m t)}{1 - \left(\frac{X_0}{X_m}\right)(1 - exp(\mu_m t))}$$

where

X_0=initial cell concentration, g/l.

The rearrangement of Eq. (2) yields Eq. (3):

$$ln\frac{X}{X_m - X} = \mu_m t - ln\left(\frac{X_m}{X_0} - 1\right)$$

The value of X_m is evident from the experimental data. A plot of ln ($X/(X_m-X)$) versus time (t) will give a line of slope equal to μ_m and intercept equal to ln ((X_m/X_0)-1), from which the initial inoculum size X_0 can be calculated.

Luedeking–Piret equation [30]

This equation correlates the product formation rate with growth rate and cell concentration as follows:

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X$$

where

dP/dt = the product formation rate.

α=the growth-associated product formation coefficient,

β=the nongrowth-associated product formation coefficient, h^{-1}.

This equation is usually expressed by dividing its two components by the biomass (*X*), which gives the specific production rate versus the specific growth as follows:

$(dP/dt)/X = \alpha\ (dX/dt)/X + \beta$

where

$((dP/dt)/X)$=specific production rate, h^{-1}.

$((dX/dt)/X)$=specific growth rate, h^{-1}.

A plot of $((dP/dt)/X)$ versus $((dX/dt)/X)$ gives a line of slope equal to α and an intercept equal to β.

Results

The data obtained from all the fermentation processes were analyzed and calculated on Microsoft Excel 2019. The data were represented in charts and the trend lines along with correlation coefficients (R^2) were shown on each chart. All the calculated R^2 showed good correlations between data.

Comparison of three levels of agitation for PHB production

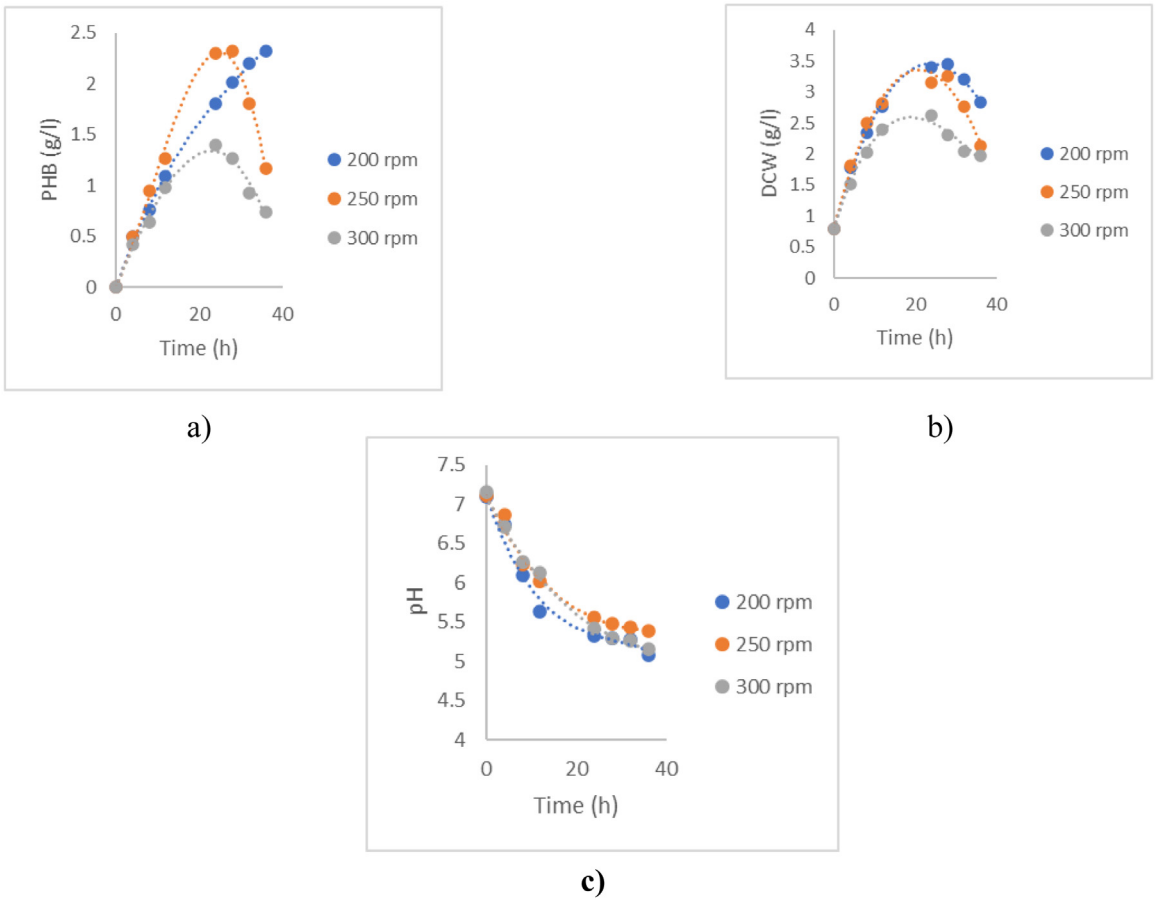
A set of fermentation batches were established in a 5 L laboratory fermenter with a working volume of 3 L to scale up PHB production using *B. aryabhattai* MH997667.1. The experiments were performed at a constant aeration rate of 1.0 vvm (volume air per volume liquid per minute) and at a temperature of 30°C with different agitation speeds of 200, 250, and 300 rpm.

A comparison of PHB concentration, dry cell weight, and pH using different agitation speeds (200, 250 and 300 rpm) is shown in Fig. 1a-c. The highest PHB yield % (73.01%) was expressed by 250 rpm at 24 h. The PHB concentration at 250 rpm was 27% and 64% higher than that at 200 and 300 rpm, respectively, whereas the biomass concentration was nearly the same at 200 and 250 rpm during the first 24 h and decreased by 16% at 300 rpm. There was no change in pH values on increasing the agitation speed. Results in Fig. 2a, b show that both specific growth rate and specific production rate were not affected by changing the agitation speed. However, the PHB yield concerning biomass (Fig. 2c) was nearly the same at the first 12 h for both 250 and 300 rpm and decreased by 30% at 200 rpm.

Kinetic parameters for PHB production

The kinetic equations for growth and product formation proposed to describe the batch fermentations for PHB production in a benchtop fermenter were logistic and Luedeking–Piret equations, respectively. The kinetic parameters were estimated on the data from batch fermentation at 250 rpm (Figs. 3 and 4) and the values of parameters

Figure 1



Time course of (a) PHB concentration, (b) dry cell weight, (c) pH in 200, 250, and 300 rpm fermentation batches for PHB production.

estimated from the slope and intercept of these straight lines (Table 1).

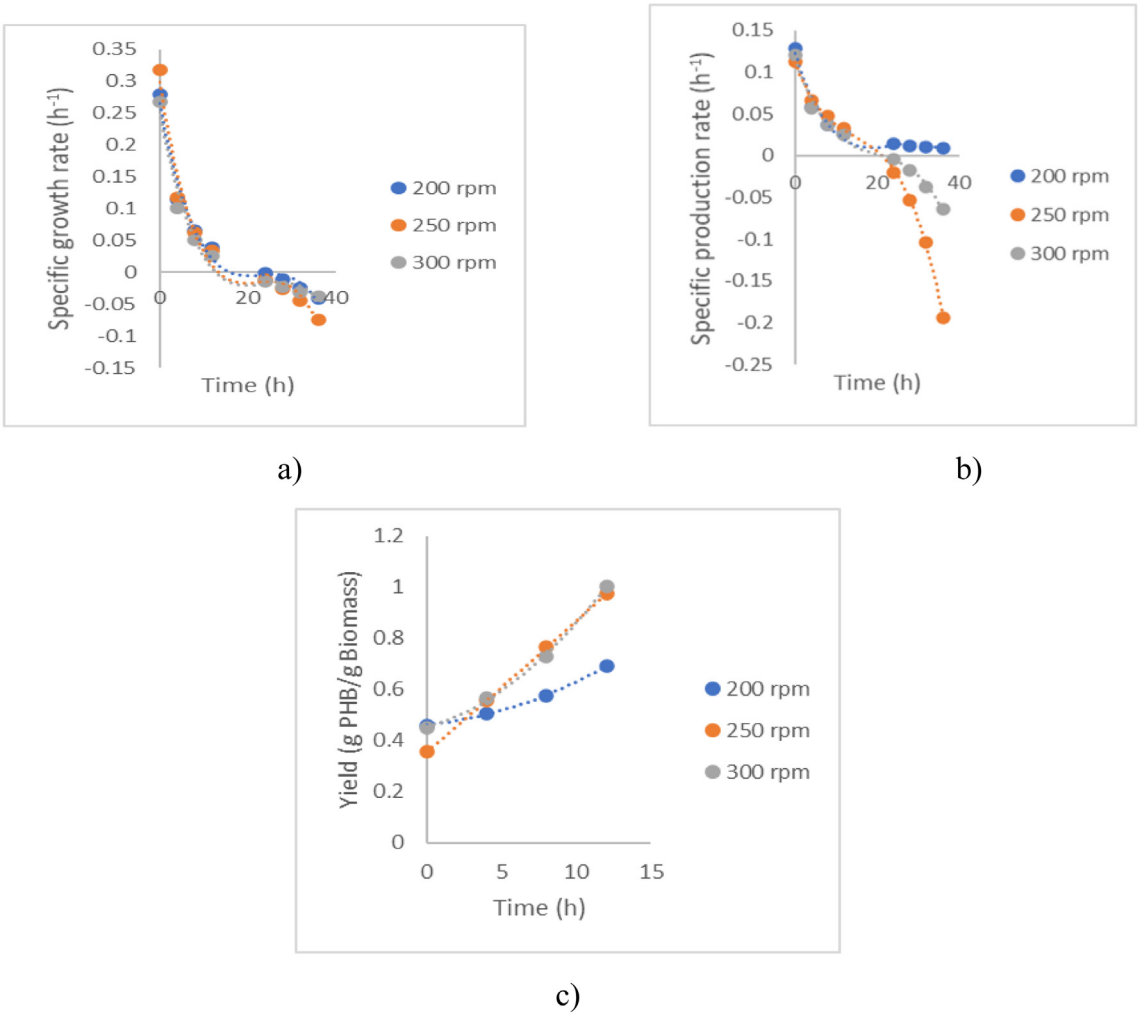
Using MATLAB software 2019 for simulating the experimental process, the biomass and PHB concentration were compared with those obtained from the simulation (Fig. 5a, b). The simulated data showed a good fit with the experimental data obtained during the first 24 h for PHB production at 250 rpm agitation speed.

Discussion

Polyhydroybutyrate (PHB) polymers are produced by different bacterial fermentation processes as the discontinuous (batch and fed-batch) and the continuous methods [31–33]. The effect of agitation speed (200, 250, and 300 rpm) on PHB production using *B. aryabhattai* MH997667.1 was studied in a batch process. The PHB yield% (73.01%) and polymer productivity (0.095 g/l/h) were significantly higher at 250 rpm agitation compared with the values obtained at 200 and 300 rpm after 24 h. The effect of varying the agitation on PHB production was evident in the maximum PHB yield obtained at 200, 250, and 300 rpm and the time required to reach those values. However, due to the continuous consumption and the decrease of the carbon source in the fermentation medium, the PHB accumulation ceased in the early stages of the stationary phase. Similar results were reported by Valappil *et al.* [3] using *B. cereus* SPV.

In this batch fermentation, the PHB yield and the productivity were nearly the same compared with the results obtained in our previous work using shaken flask cultures [25], but in a shorter time of fermentation (24 h in batch culture and 36 h in shaken flasks). However, the agitation speed had almost no effect on biomass concentration at lower values (200 and 250 rpm), while the higher agitation (300 rpm) affected the biomass inversely. This was in agreement with Lasemi *et al.* [34] where they obtained maximum values of biomass (10.1 g/l) and PHB concentration (3.6 g/l) at 250 rpm after 15 h. On the contrary, other researchers [35] have studied the influence of agitation speed on PHB production using different strains and found that there was no effect at lower levels of agitation (5.8 g/l PHB at 150 rpm),

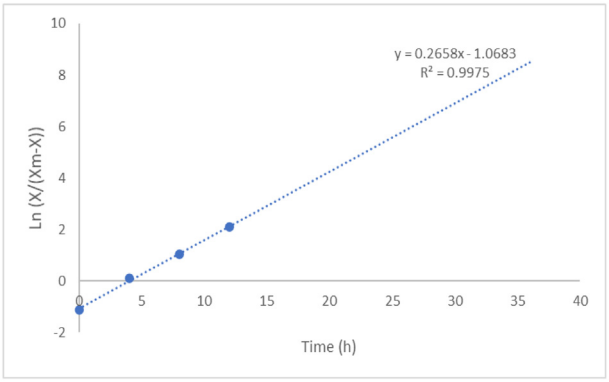
Figure 2



Time course of (a) specific growth rate, (b) specific production rate, (c) PHB yield in 200, 250, and 300 rpm fermentation batches for PHB production.

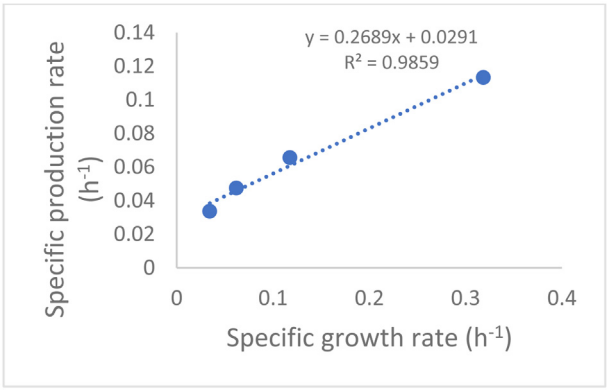
whereas at higher levels of agitation, the PHB produced decreased. This was attributed to the fact that the mixing process affected the mass transfer of oxygen, which in turn facilitated both the polymer formation and biomass growth once the sufficient oxygen content was achieved. However, the higher speeds of agitation (more than 250 rpm) increase the shear rate due to the formation of intracellular granules

Figure 3



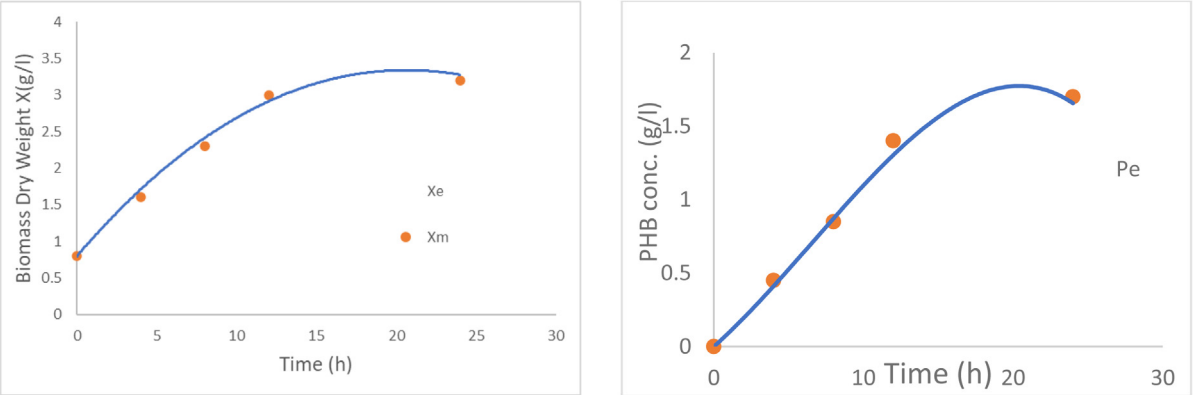
Logistic kinetic model fitted with the experimental results from a batch fermentation process using 250 rpm.

Figure 4



Luedeking–Piret kinetic model fitted with experimental results from a batch fermentation process using 250 rpm.

Figure 5



Comparison between the experimental and simulated data of (a) biomass concentration and (b) PHB production in a batch process using 250 rpm.

Table 1 Estimated kinetic parameters

μ_m (maximum specific growth rate, h^{-1})	0.2658
X_0 (initial cell concentration, g/l)	0.8
X_m (maximum cell concentration, g/l)	3.2
α (growth-associated product formation coefficient)	0.2689
β (nongrowth-associated product formation coefficient, h^{-1}.)	0.0291

[34,35]. Similarly, Zahari *et al.* [36] also reported that the PHB concentration increased proportionally by increasing the agitation speed up to 220 rpm after which the PHB decreased. It was also observed that there was no effect on PH at various agitation speeds. The mathematical model proposed for the batch fermentation described accurately the biomass and PHB formation versus the time.

Conclusion

This study presents the production of PHB by the fermentation of *B. aryabhattai* MH997667.1 in a 5L-laboratory fermenter. Different agitation speeds were tested and the experimental results were compared with our results previously attained using shaken flask cultures. In the batch mode, nearly the same biomass, PHB productivity, and PHB concentration were achieved but in a shorter time of fermentation. The mean experimental data for the batch cultivation were used to predict kinetic parameters. The logistic equation was the best model that predicted well the biomass growth (R^2=0.9975), while the Luedeking–Piret equation was the best model to describe the PHB concentration (R^2=0.9859).

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Nil.

Conflicts of interest

The authors declare no conflict of interest.

References

1 Anderson AJ, Dawes EA. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Mol Biol Rev 1990; 54:450–472.

2 Yanti NA, Sembiring L, Margino S, Ahmad SW. Bacterial production of poly-β-hydroxybutyrate (PHB): converting starch into bioplastics. Bioplastics Sustain Dev 2021; 259–276.

3 Valappil SP, Misra SK, Boccaccini AR, Keshavarz T, Bucke C, Roy I. Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterised Bacillus cereus SPV. J Biotechnol 2007; 132:251–258.

4 Shokr A, Abd-Elsalam O, Hamedo H, Elshafey N. Isolation and identification of Polyhydroxybutyrate (PHB)-producing microorganisms from various sources in North Sinai. Catrina: Int J Environ Sci 2023; 27:23–31.

5 Jaiganesh R, Tattwaprakash C, Kumar GR, Iyappan S, Jaganathan MK. Characterization and Optimization of process parameter for maximum Poly (-β-) hydroxyl butyrate production by bacteria isolated from the soil. Res J Pharm Technol 2019; 12:4926–4930.

6 Kumar S, Sharma MK, Sharma MK, Kumar G. Isolation of local strain of Bacillus sp. SM-11, producing PHB using different waste raw as substrate. Res J Pharm Technol 2022; 15:2053–2058.

7 Alshehrei F. Production of polyhydroxybutyrate (PHB) by bacteria isolated from soil of Saudi Arabia. J Pure Appl Microbiol 2019; 13:897–904.

8 Reddy AR, Kumar RB, Prabhakar KV. Isolation and identification of PolyHydroxyButyrate (PHB) producing bacteria from Sewage sample. Res J Pharm Technol 2017; 10:1065–1069.

9 Shodhganga T. Review of literature on poly-3hydroxybutyrate. A PhD thesis submitted to Department of Environmental and Sustainability Studies, Teri University, Vasant Kunj, India. 2011; 6–52.

10 Adnan M, Siddiqui AJ, Ashraf SA, Snoussi M, Badraoui R, Alreshidi M, *et al.* Polyhydroxybutyrate (PHB)-based biodegradable polymer from Agromyces indicus: enhanced production, characterization, and optimization. Polymers 2022; 14:3982.

11 Khanna S, Srivastava AK. Statistical media optimization studies for growth and PHB production by Ralstonia eutropha. Process Biochem 2005; 40:2173–2182.

12 Deepa S, Bhuvanehswari P, Kanimozhi K, Panneerselvam A. Utilization of industrial waste for the production of poly-beta-hydroxy butyrate (Phb) by Alcaligenes eutrophus. Res J Sci Technol 2013; 5:421–424.

13 Pratap JK, Krishnan K. Microbial production of Polyhydroxyalkonates (Bioplastic) using cheap household waste resources and their biomedical applications: a systematic review. Lett Appl NanoBioScience; 2023; 12:174.

14 Kim BS. Production of poly (3-hydroxybutyrate) from inexpensive substrates. Enzyme Microb Technol. 2000; 27:774–777.

15 Ghaly AE, Tango MA, Adams MA. Enhanced lactic acid production from cheese whey with nutrient supplement addition. Agric Eng Int: CIGR J 2003; 5:1–20.

16 Sharifzadeh BM, Tabandeh F, Yunesi M, Najafpour G, Issazadeh H, Khodabandeh M. Optimization Phas production by Cupriavidus necator from molasses and acetate as substrate. N Biotechnol 2009; 25:268–272.

17 Koutinas AA, Xu Y, Wang R, Webb C. Polyhydroxybutyrate production from a novel feedstock derived from a wheat-based biorefinery. Enzyme Microb Technol 2007; 40:1035–1044.

18 Oliveira FC, Dias ML, Castilho LR, Freire DM. Characterization of poly (3-hydroxybutyrate) produced by Cupriavidus necator in solid-state fermentation. Bioresour Technol 2007; 98:633–638.

19 Gouda MK, Swellam AE, Omar SH. Production of PHB by a Bacillus megaterium strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. Microbiol Res 2001; 156:201–207.

20 Byrom D. Polymer synthesis by microorganisms: technology and economics. Trends Biotechnol 1987; 5:246–250.

21 El-Metwally NM, Abd El-Khattab NA, Shafei MS, Allam RF, Ragab YM, Amin MA. Cloning of Acetoacetyl-CoA reductase and Polyhydroxybutyrate synthase genes from the local isolate Bacillus aryabhattai 6N-NRC into Escherichia coli. Res J Pharm Technol 2021; 14:3299–3306.

22 Shahhosseini S. Simulation and optimisation of PHB production in fed-batch culture of Ralstonia eutropha. Process Biochem 2004; 39:963–969.

23 Mahmoudi M, Baei MS, Najafpour GD, Tabandeh F, Eisazadeh H. Kinetic model for polyhydroxybutyrate (PHB) production by Hydrogenophaga pseudoflava and verification of growth conditions. Afr J Biotechnol 2010; 9:3151–3157.

24 Divyashree MS, Rastogi NK, Shamala TR. A simple kinetic model for growth and biosynthesis of polyhydroxyalkanoate in Bacillus flexus. N Biotechnol 2009; 26:92–98.

25 El-Metwally NM, Allam RF, Shafei MS, Amin MA, Ragab YM, El-Refai HA, Khattab AENA. Optimization of Polyhydroxybutyrate (PHB) production by locally isolated Bacillus aryabhattai using response surface methodology. Curr Trends Biotechnol Pharm 2020; 14:359–376.

26 Du G, Si Y, Yu J. Inhibitory effect of medium-chain-length fatty acids on synthesis of polyhydroxyalkanoates from volatile fatty acids by Ralstonia eutropha. Biotechnol Lett 2001; 23:1613–1617.

27 Law JH, Slepecky RA. Assay of poly-β-hydroxybutyric acid. J Bacteriol 1961; 82:33–36.

28 Nehra K, Jaglan A, Shaheen A, Yadav J, Lathwal P, Singh M. Production of Poly-β-Hydroxybutyrate (PHB) by bacteria isolated from rhizospheric soils. Int J Microb Resour Technol 2015; 2:38–48.

29 Moraine RA, Rogovin P. Kinetics of polysaccharide B-1459 fermentation. Biotechnol Bioeng 1966; 8:511–524.

30 Luedeking R, Piret EL. A kinetic study of the lactic acid fermentation. Batch process at controlled pH. J Biochem Microbiol Technol Eng 1959; 1:393–412.

31 Koller M. A review on established and emerging fermentation schemes for microbial production of polyhydroxyalkanoate (PHA) biopolyesters. Fermentation 2018; 4:30.

32 Li M, Wilkins M. Fed-batch cultivation and adding supplements to increase yields of polyhydroxybutyrate production by Cupriavidus necator from corn stover alkaline pretreatment liquor. Bioresour Technol 2020; 299:122676.

33 Nygaard D, Yashchuk O, Noseda DG, Araoz B, Hermida ÉB. Improved fermentation strategies in a bioreactor for enhancing poly (3-hydroxybutyrate)(PHB) production by wild type Cupriavidus necator from fructose. Heliyon 2021; 7:e05979.

34 Lasemi Z, Darzi GN, Baei MS. Media optimization for poly (β-hydroxybutyrate) production using Azotobacter Beijerinckii. Int J Polym Mater Po 2013; 62:265–269.

35 Musa H, Bolanle BB, Kasim FH, Arbain D. Screening and production of polyhydroxybutyrate (PHB) by bacterial strains isolated from rhizosphere soil of groundnut plants. Sains Malays 2016; 45:1469–1476.

36 Zahari MAKM, Ariffin H, Mokhtar MN, Salihon J, Shirai Y, Hassan MA. Factors affecting poly (3-hydroxybutyrate) production from oil palm frond juice by Cupriavidus necator (CCUG52238T). J Biomed Biotechnol 2012; 2012:1–8.