Statistical optimization for polyhydroxybutyrate production by locally isolated *Bacillus safensis* using sugarcane molasses under nutritional stressed conditions

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Background and objective
Biodegradable polymers, such as polyhydroxyalkanoate (PHAs), have recently been recognized as polyesters owing to their properties as biodegradable thermoplastics.
Materials and methods
The main objective of this study was to isolate an efficient polyhydroxybutyrate (PHB) producer from soil collected from different rhizospheric areas in Egypt. The potent strain was identified using 16 s rRNA sequence analysis. Plackett–Burman and central composite sequential designs were used to investigate cultural variables influencing PHB production (central composite sequential design).
Results and conclusion
Of a total of nine different isolates, three were found to be PHB positive based on the color using Nile Red stain. The potent strain was identified as *Bacillus safensis* (NR_113945.1). Using one factor at a time experiments, sugar molasses and ammonium sulfate, respectively, were the best carbon and nitrogen sources, whereas the best inoculum was 10 ml/100 ml of fermentation medium. Cultural variables influencing PHB production were studied using Plackett–Burman and central composite sequential designs. Accordingly, the most influential factors on PHB production were sugarcane concentration, inoculum size, and KH_2PO_4. Under the optimized conditions, a PHB content (93% cell dry weight) of 1.17 folds was attained. Furthermore, the Fourier transform infrared spectroscopy and ^{1}H-NMR results confirmed the produced polymer as PHB. These results give insight into the use of locally isolated bacteria (*B. safensis* (NR_113945.1) utilizing inexpensive substrate such as sugarcane molasses for PHB production.

Keywords:
Bacillus safensis, polyhydroxyalkanoate, statistical optimization, sugarcane molasses

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Introduction

Synthetic polymers are toxic environmental pollutants as they are nondegradable and thus accumulate in large quantities everywhere. Recently, biodegradable polymers such as polyhydroxyalkanoate (PHAs) were recognized as polyesters due to their features as biodegradable thermoplastics [1].

Previously, 150 constituents of PHAs have been successfully identified, leading to a great variety of their properties [2]. Polyhydroxybutyrates (PHBs), a derivative of PHAs, have received a lot of attention because their structural features are similar to those of classic petroleum plastics like polypropylene and polyethylene [3,4]. PHB is mostly produced by prokaryotes and accumulated intracellularly as reserve granules that are used as sources for carbon-energy storage, which are in turn used as a sink for carbon and reducing equivalents [5,6]. It is a carbon assimilation product that microorganisms utilize as an energy storage molecule [7,8]. PHB differs from other biodegradable plastics in that it is water insoluble, extremely resistant to hydrolytic destruction, ultraviolet resistant, oxygen permeability, low acid and basic resistance, chloroform insoluble, and biocompatible, making it ideal for medical applications [9]. These biodegradable polyesters display a special interest as they can be used as plastic alternatives and totally degraded by the microorganisms in the environment while at the same time being easily produced from regenerable carbon sources [10,11]. PHB is commonly found in different bacterial genera such as *Bacillus*, *Azotobacter*, *Cupriavidus*, *Pseudomonas*, *Ralstonia*, and *Rhizobium* [12]. The expense of producing PHB is a key barrier to

replacing petroleum-based plastics with bioplastics, so numerous solutions have been offered, including the use of waste products and an optimization approach to PHB production [4,13]. Several strategies have been applied for the optimization of medium composition as well as the physical factors affecting PHB production. These strategies include either one factor at a time or statistical optimization adopting response surface methodology (RSM) [4,14]. The traditional optimization of PHB yield takes a long time and ignores the interactions between different chemicals and physical conditions. RSM, on the contrary, can assist in understanding and exploring the link between various factors based on their responses [15].

This research was designed to use a local bacterial isolate that was selected and identified and also examined its efficiency to utilize readily available and cheap carbon sources to produce PHB. Moreover, it focuses on the optimization of various growth and physiological factors using surface methodology to increase the PHB yield.

Materials and methods

Isolation and screening of polyhydroxyalkanoate producing bacteria

Screening of PHB-producing microorganisms was done by viable-colony staining method using Nile Red stain as follows: soil samples were collected from El-Minea, Marsa Alam, and Red Sea regions. One gram of each soil samples was serially diluted to 10^{-7} in sterile distilled water. Each dilution was cultured on sterilized minimal salt agar media containing 0.25 mg/100 ml Nile Red stain. After 48 h of incubation at 30°C, colonies with pinkish pigment that indicated PHB production were exposed to ultraviolet light (312 nm) to detect the accumulation of PHB according to the lighted plates and were given positive signs. After that these isolates were picked up and purified by subculturing on the same media [16,17].

Molecular identification

XXXX16S rRNA gene sequencing and analysis

DNA was extracted by Wizard1 Genomic DNA purification kit supplied by Promega (Promega, Southampton, UK) according to the manufacturer's guidelines. PCR amplification was done using Maxima Hot Start PCR Master Mix (Thermo K1051) in Sigma Company of Scientific Services, Egypt (www.sigma-co-eg.com). For phylogenetic analysis, the determined sequences were compared with the sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov) by BLAST search.

Determination of cell dry weight

Culture medium was collected after 48 h of incubation at 30°C and the cell dry weight was measured by centrifugation of 100 ml of the culture at 10 000 rpm for 15 min at 4°C. The supernatant was discarded, and the bacterial cells were washed twice in deionized water and dried at 80°C until a constant weight and then the total bacterial cell dry weight was determined as g/l [18].

Fermentation and production medium

Minimal salt medium (MSM) was prepared as follows: g/l KH_2PO_4: 1.5 g; Na_2HPO_4: 3.525 g; $MgSO_4.7H_2O$: 0.2 g; $CaCl_2$: 0.02 g; ferric citrate: 0.0015 g; glucose: 20 g; NH_4Cl 0.75 g; trace elements solution 1 ml; distilled water 1000 ml; and pH 7.5 for the production of poly-β-hydroxybutyrate. MSM (100 ml) was taken in each Erlenmeyer flask and autoclaved at 121°C for 20 min after which 10% (v/v) of fresh bacterial inoculum was inoculated in each flask and incubated for 7 days at 30°C [19].

Extraction and quantification of polyhydroxybutyrate

All the PHB-positive bacterial isolates were inoculated in MSM, and the cell growth of each isolate containing the polymer was centrifuged at 10 000 rpm at 4°C for 10 min The biomass was resuspended in equal volume of 4% sodium hypochlorite and incubated at 37°C for 24 h. The mixture was then centrifuged at 10 000 rpm for 10 min to sediment the lipid granules. The supernatant was discarded, and the cell biomass was washed successively with water, acetone, and ethanol to remove unwanted materials. The whole mixture was centrifuged again and the supernatant was discarded. Finally, the purified material was dissolved in hot chloroform and filtered through Whatman no. 1 filter paper. To the filtrate, 10 ml of hot concentrated H_2SO_4 was added, which converts the polymer to crotonic acid, turning it into a brown colored solution. The solution was cooled and absorbance was read at 235 nm against a concentrated H_2SO_4 blank on a ultraviolet–visible spectrophotometer. The quantity of PHB produced was determined by referring to the standard curve [20,21].

Optimization of cultural parameters for maximum polyhydroxybutyrate production

Effect of different incubation periods on polyhydroxybutyrate production

MSM was sterilized at 121°C for 20 min, and then flasks were inoculated with a 10% v/v bacterial

suspension and incubated at 30°C at 200 rpm at different incubation times (24, 48,72, 96, 120, 144, and 168 h). The dry weight of the cell cultures and the yield of PHB were measured at each time point.

Effect of different inoculum periods

Each 250 ml Erlenmeyer flask containing 100 ml medium was inoculated with 10 ml of inoculum of 4-, 12-, 24-, 48-, and 72-h-old cultures. After 48 h of incubation at 30°C, the PHB yield was determined, and the optimum inoculum age was selected.

Effect of different carbon sources

The effect of different carbon sources on PHB production was determined by inoculating the bacterial isolate in 100 ml of MSM supplemented with different carbon sources such as glucose, lactose, sucrose, and mannose at 2% concentration. Cheap waste as carbon sources like sugarcane, beet molasses, fried oil, and wheat bran were also tested as carbon sources. Cultures were incubated at 30°C on a rotary shaker (200 rpm) for 48 h. After incubation, PHBs produced were quantified spectrophotometrically as described before.

Effect of different nitrogen sources

The selected bacterial isolate was grown in 100 ml of MSM broth containing the optimum carbon source, and different organic and inorganic nitrogen sources (ammonium sulfate, ammonium chloride, urea, yeast extract, corn steep liquor, tryptone, and peptone) were used at nitrogen base concentration. After 48 h of incubation at 30°C, the PHB yield was determined, and the optimum nitrogen source was selected.

Screening of most significant production parameters by Plackett–Burman design

Screening of the most effective parameters affecting PHB production was studied by Plackett–Burman design [22]. Statistical software package Minitab software, version 16.1 (2010; Minitab Inc., State College, Pennsylvania, USA) was used to evaluate the most important physical and nutritional factors for PHB synthesis. A total of six variables were included: sugarcane molasses (x_1), ammonium sulfate (x_2), inoculum size (x_3), time (x_4), disodium hydrogen phosphate (x_5), and potassium dihydrogen phosphate (x_6) (Table 2). All the trials were completed in duplicate. Each variable represented at three levels (high +, low −, and medium 0). Each row represented the number of the trial, whereas each column represented an independent variable.

Central composite sequential design

After stating the significant factors for PHB production by *B. safensis* through PBD, a central composite sequential design was designed to optimize the significant variables [sugar cane molasses (x_1), inoculum size (x_2), and potassium dihydrogen phosphate (x_3)]. The three independent variables were studied at five levels. The statistical software package used was Minitab software, version 16.1 (Minitab Inc.).

Results and discussion

Isolation and screening of isolates for polyhydroxybutyrate production

Under constrained conditions of nitrogen and phosphorous in a surplus of carbon supply, a wide range of bacteria have been found that make PHA granules. According to various studies, high-density PHB formation is caused by the depletion of critical nutrients such as nitrogen, phosphorus, and magnesium in the presence of an excess carbon supply, which aids in the metabolism shifting from growth to PHB production [23,24].

PHB is the most leading compound of the PHA group, as it can be easily consumed in the environment by different microorganisms using PHB polymerase to nontoxic products [25].

The goal of this study was to examine three samples collected from diverse environmental locations in Upper Egypt (El-Minea and Marsa Alam). As previously stated, the bacterial isolates were purified and stored on their appropriate media for further research. PHB synthesis was investigated in a total of nine isolates. The lipophilic dye Nile red, which reacted with phospholipids found in the outer layer of PHB, was used to identify the active PHB producers [26].

Three of the nine bacterial isolates were capable of growing extensively on MSM supplied with 2% glucose and Nile red stain, indicating good PHB synthesis action. The most efficient PHB producers exhibited pinkish color owing to the presence of PHB granules (Fig. 1). To confirm these findings, these bacterial isolates were further measured quantitatively in different time intervals (48, 96, and 120 h) to choose the best isolate (data not shown). Of three isolates, E1, which was isolated from rhizopheric soil in Upper Egypt, mainly El-Menia, expressed the maximum PHB production (0.4 g/l, 40% CDW) after 48 h.

Molecular identification

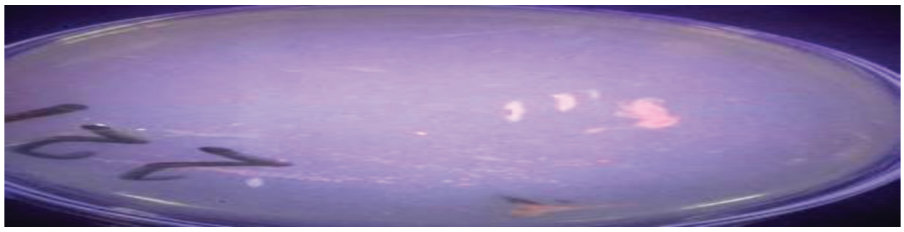
From the genomic DNA of isolate E1, the entire 16 S rRNA gene fragment (1000 bp) was amplified. The 16 S rRNA sequence revealed maximum similarity with *Bacillus* sp. The phylogenetic analysis proved that the active isolate for PHB production is *B. safensis* (NR_113945.1) with 97.76% identity (Fig. 2). This is the first research of *B. safensis* (NR 113945.1) for PHB production that we are aware of. Previous results have reported that *Bacillus sp.* is an ideal PHB producer [27].

Characteristics of extracted polymer

Fourier transform infrared spectroscopy

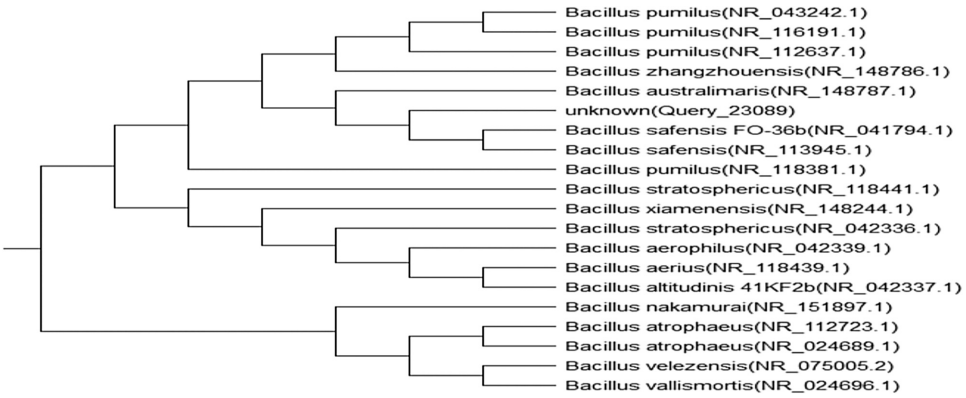
IR spectra in the range of 4000–5000 cm^{-1} were recorded using a polymer extracted from *B. safensis* (NR 113945.1). The absorption bands at 1721 and

Figure 1



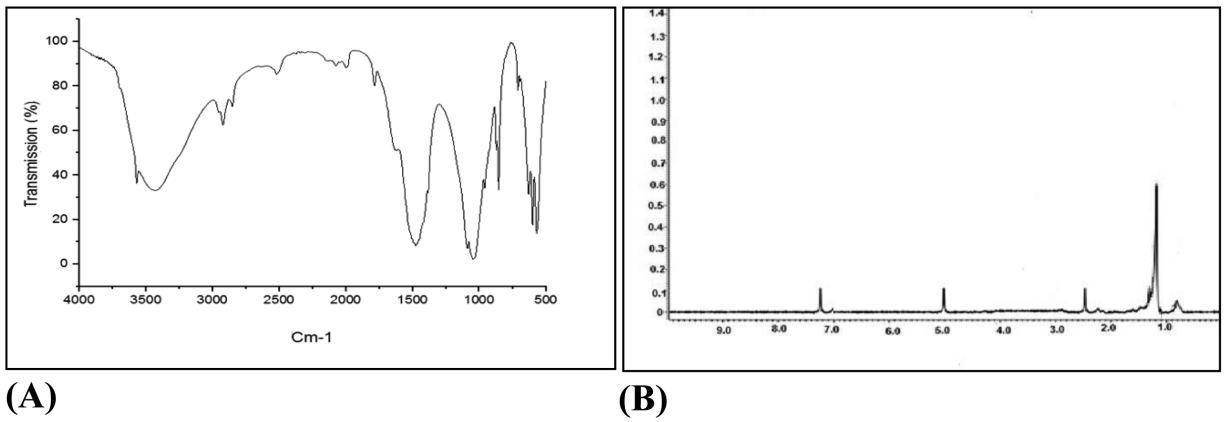
The PHA-producing isolate (E1) under ultraviolet light. PHA, polyhydroxyalkanoate.

Figure 2



Neighbor-joining tree based on the 16 S rRNA gene sequences showing phylogenetic relationship between strains E1 and closely related to the genus *Bacillus*.

Figure 3



(a) FTIR and (b) ^{1}H-NMR resonance spectra of the PHB extracted from *Bacillus safensis* (NR_113945.1). PHB, polyhydroxybutyrate; FTIR, Fourier transform infrared spectroscopy.

1000–1300 cm^{-1} in the IR spectra (Fig. 3a) were found to be unique for C<td:glyph name="dbnd"/>O and C-O stretching vibrations, respectively. These peaks were comparable with the standard peaks of 1728–1740 cm^{-1} for short and medium PHA, respectively [28]. The C-H stretching vibrations of the methyl and methylene groups are responsible for the absorption bands at 2975 and 2933 cm^{-1}, respectively. The structure of PHB is confirmed by these significant absorption bands [29,30].

^{1}H-NMR spectroscopy

The structure of the PHBs by *B. safensis* (NR_113945.1) was verified using ^{1}H-NMR spectroscopy as shown in Fig. 3b. The major peaks in the NMR spectra were 1.2, 2.4, and 5.0 ppm, which are significant for the PHB structure. The peak at 1.2 ppm resulted from the resonance absorption of methyl group (CH_3), whereas the series of peaks at 2.4 ppm proved the incorporation of methylene group (CH_2). Moreover, the peak at 5.0 ppm corresponds to the methane group (CH). These findings are in accordance with the previous reports [4,31,32].

Optimization of the cultural parameters for polyhydroxybutyrate production

Different incubation period

B. safensis (NR_113945.1) was chosen for further studies as it produced maximum PHB. The time course analysis showed that PHB was a growth-associated product (Fig. 4). Maximum values of PHB were achieved at 2 days of incubation and 40% of the CDW. After 3 days, there was a decrease in the PHB content until the yield reached 6.9% CDW. Thus, the synthesis of PHB was noticed from the log phase of growth, and it continued till the late exponential phase. This may be due to the nutrient depletion and cell consumption of PHB as an energy and carbon source to maintain its metabolic activities [33,34]. Similar results were recorded by Valappil *et al.* [35] In contrast, Ramadas *et al.* [36] reported that once the maximum PHB concentration was achieved, the PHB produced was not degraded to be used for sporulation.

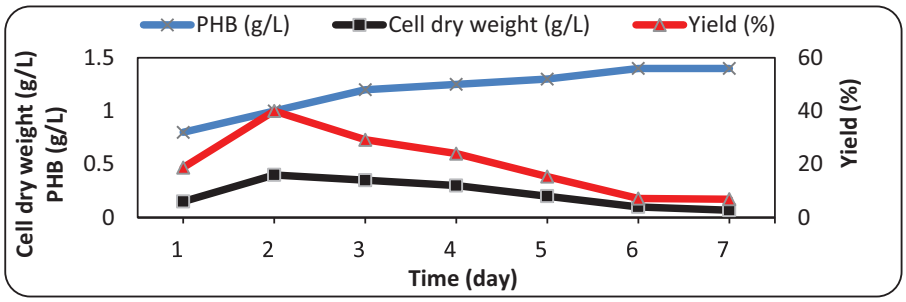
Effect of inoculum age

The pre-inoculum (24 h age) that was prepared in the MSM medium increased the biomass and showed maximum PHB production with a yield of 53.33% CDW (Fig. 5). This may be owing to when these cells were placed in the fermentation medium, easy assemble carbon source facilitated the growth of the bacterial isolate E1.

Effect of using different carbon sources on polyhydroxybutyrate production

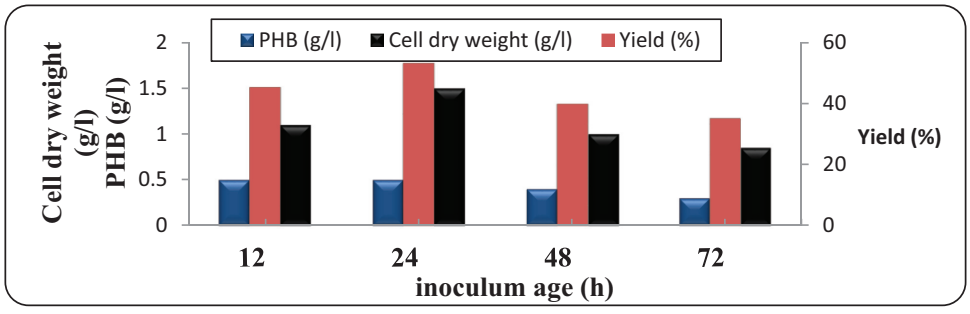
The synthesis of PHB is dependent on the type of the carbon source utilized by the bacteria. In the present

Figure 4



Growth and PHB accumulation of the newly isolated. PHB, polyhydroxybutyrate.

Figure 5



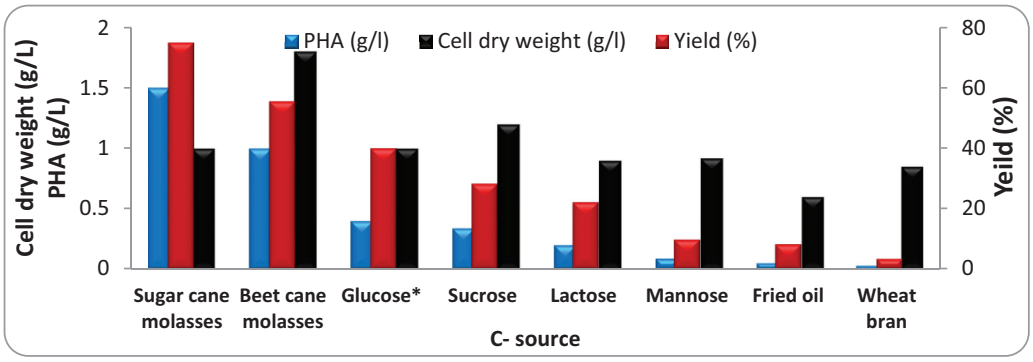
Effect of different inoculum age on PHB production. PHB, polyhydroxybutyrate.

study, the culture was grown for 24 h at 37°C and the effects of different cheap carbon sources (sucrose, lactose, and mannose) and waste (fried oil, wheat bran, sugarcane molasses, and beet molasses), which were substituted in equimolar amounts of glucose, were assessed, and the PHB % was calculated (Fig. 6). Among the tested carbon sources, sugarcane molasses was found to be the preferred substrate for maximum PHB accumulation (yield 75% CDW). However, the bacterial isolate also showed good polymer yield and cell dry weight (55.5% CDW and 1.8 g/l, respectively) with beet molasses as the carbon source. This effect could be attributed to the waste components' combination of carbon sources. These findings may aid in lowering the cost of PHB production, which is critical in the biotechnological area for fermentation operations [37]. The role of carbon sources within the microorganism is the biomass synthesis, maintenance of the cell, and PHA polymerization [38]. Sugarcane molasses contains trace elements and vitamins such as riboflavin, pyridoxine, and thiamin; therefore, it can be used as a source of growth factors [39]. In addition, several agro-industrial residues such as potato starch, soycake, cane molasses, and whey have been reported to reduce the PHB production cost [40,41].

Effect of using different nitrogen sources on polyhydroxybutyrate biosynthesis

The effect of nitrogen was studied to select the most effective nitrogen source for maximum PHB biosynthesis (Fig. 7). Various nitrogen sources (using 0.075% ammonium chloride as a control on an equal nitrogen basis) were added in the MSM medium with the best carbon source (sugarcane molasses 2%), and the PHB yield and the cell dry weight were depicted. Ammonium sulfate as an inorganic nitrogen source was found to be the best nitrogen source, where the PHB yield was 79.16 of cell dry weight. The next promising nitrogen source was ammonium chloride, showing 75%

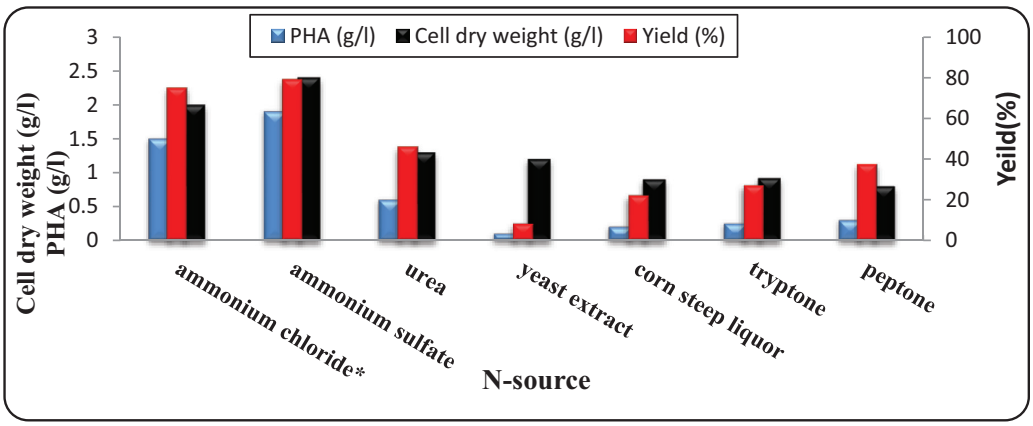
Figure 6



*control

Effect of different carbon sources on PHB production. PHB, polyhydroxybutyrate.

Figure 7



*control

Effect of different nitrogen sources on PHB production. PHB, polyhydroxybutyrate.

Table 1 Plackett–Burman experimental design matrix selection significant variables of polyhydroxybutyrate production by *Bacillus safensis*

Trial no	Sugarcane molasses (g/l) X_1	Ammonium sulfate (g/l) X_2	Inoculums size (ml/l) X_3	Time (days) X_4	Disodium hydrogen phosphate(g/l) X_5	Potassium dihydrogen phosphate (g/l) X_6	Yield (%)
1	–(15)	+(0.8)	+(15)	0(1)	–(3)	+(2)	6.6
2	+(25)	–(0.2)	–(5)	–(1)	–(3)	+(2)	22
3	–(15)	+(0.8)	–(5)	–(1)	+(5.8)	–(1)	20
4	+(15)	–(0.2)	–(5)	+(3)	+(5.8)	–(1)	10
*5	0 (20)	0 (0.5)	0 (10)	0 (2)	0 (4.4)	0 (1.5)	80
6	–(15)	–(0.2)	+(15)	+(3)	–(3)	–(1)	87.5
7	+(25)	– (0.2)	+(15)	1(1)	+(5.8)	–(1)	93
8	+(25)	+(0.8)	–(5)	+(3)	–(3)	–(1)	
9	+(25)	+(0.8)	+(15)	+(3)	+(5.8)	+(2)	

Table 2 Central composite sequential design of independent variables for polyhydroxybutyrate production by *Bacillus safensis*

Run	Sugarcane molasses X_1	Inoculum size (ml) X_2	KH_2PO_4 X_3	Yield (%)
1	20 (0)	10 (0)	2.34 (++)	25
2	15 (–)	5 (–)	1 (–)	20
3	20 (0)	10 (0)	1.5 (0)	80
4	20 (0)	1.59 (– –)	1.5 (0)	15
5	15 (–)	15 (+)	1 (–)	87.5
6	20 (0)	10 (0)	1.5 (0)	75.2
7	20 (0)	10 (0)	1.5 (0)	70
8	15 (–)	15 (+)	2 (+)	6.6
9	15 (–)	15 (+)	2 (+)	10
10	25 (+)	15 (+)	1 (–)	93
11	11.59 (– –)	10 (0)	1.5 (0)	45.1
12	25 (+)	15 (+)	2 (+)	6.8
13	25 (+)	5 (–)	2 (+)	22
14	20 (0)	10 (0)	1.5 (0)	72.8
15	20 (0)	10 (0)	1.5 (0)	77.4
16	25 (+)	5 (–)	1 (–)	76.7
17	20 (0)	18.40 (++)	1.5 (0)	66.4
18	20 (0)	10 (0)	1.5 (0)	70.5
19	28.40 (++)	10 (0)	1.5 (0)	85.3
20	20 (0)	10 (0)	0.65 (– –)	95

PHB yield. Yeast extract was found to be the least supporter for PHB biosynthesis. These results agreed with Khanna and Srivastava [10] who reported that ammonium sulfate was the best nitrogen source for the optimization of PHB. Similarly, Lathwal *et al.* [21] found that the highest PHB yield was achieved on using ammonium sulfate followed by ammonium chloride. Because it is a simple nitrogen source, ammonium sulfate is likely to be more readily available than other complexity nitrogen sources.

Statistical experimental design for evaluation of factors influencing polyhydroxybutyrate synthesis

Plackett–Burman design

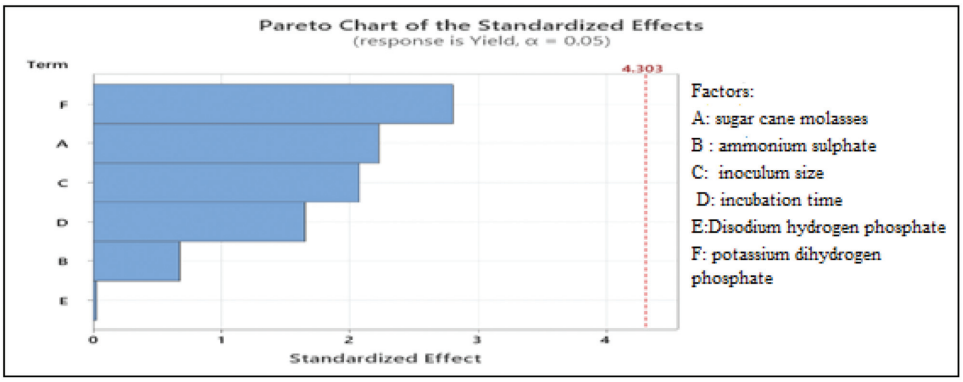
Six variables including sugarcane molasses, ammonium sulfate, inoculum size, time, disodium hydrogen phosphate, and potassium dihydrogen phosphate were analyzed for their effects on PHB production. The design has nine runs with three levels for each factor. The statistical design helps to study the individual and the various factors on PHB production using *B. safensis* (NR_113945.1) isolate. Maximum PHB yield was 93% in trial number 7 (Table 1). In the Pareto chart, moving along the *x* and *y* axes demonstrated that increasing the sugarcane molasses concentration had a conspicuous effect on PHB accumulation (Fig. 8). Increasing the inoculum size also had a positive effect on the polymer production, whereas KH_2PO_4 had a negative effect. This means that PHB accumulated under phosphate limitation. In this connection, Cavaillé *et al.* [42] reported that PHB production was enhanced when growth was restricted owing to lack of phosphorous. The analysis of variance (ANOVA) for PHB production showed that the model *F* value and the *P* value less than 0.05 and R^2 91.03% implies the analysis is significant. Thus, the optimal conditions for maximum PHB production were sugarcane molasses, 25 g/l; ammonium sulfate, 0.2 g/l; inoculum size, 15 ml; Na_2HPO_4, 5.8 g/l; and KH_2PO_4, 1 g/l after 3 days of fermentation at pH 7.0.

Optimization of polyhydroxybutyrate production using central composite sequential design

A central composite sequential design was performed to determine the optimal levels and the interactions among the selected significant parameters. In this study, a total of 20 experiments with different concentrations of sugarcane molasses (x_1), inoculum size (x_2), and KH_2PO_4 (x_3) were carried out each variable at five different levels (Table 2). The data showed great variation in the PHB production. Run numbers, 3, 5, 10, 19, and 20 showed a high PHB yield (≥80%).

Maximum polymer production (93%) was achieved at run number 20 in the presence of 29 g/l sugarcane

Figure 8



Pareto graph showing standerdized effect of different variables on PHB production by *Bacillus safensis* based on Plackett–Burman design. PHB, polyhydroxybutyrate.

Table 3 Results of analysis of variance analysis for optimization of polyhydroxybutyrate production by *Bacillus safensis*

Term	Coefficient	SE Coefficient	95% CI	*t* value	*P* value	*F* value
Constant	74.51	5.07	(63.22, 85.80)	14.70	0.000	
X_1	14.88	3.36	(7.39, 22.37)	4.43	0.001*	19.59
X_2	15.59	3.36	(8.09, 23.08)	4.64	0.001*	21.49
X_3	–20.99	3.36	(–28.48, –13.50)	–6.24	0.000*	38.97
X_1^2	–4.49	3.27	(–11.78, 2.80)	–1.37	0.200	1.88
X_2^2	–13.15	3.27	(–20.44, –5.86)	–4.02	0.002*	16.14
X_3^2	–6.50	3.27	(–13.80, 0.79)	–1.99	0.075	3.95
X_1X^2	–0.23	4.39	(–10.01, 9.56)	–0.05	0.960	0.00
X_1X_3	1.40	4.39	(–8.39, 11.19)	0.32	0.757	0.1
X_2X_3	–5.15	4.39	(–14.94, 4.64)	–1.17	0.268	1.37

s 12.428; R^2 90.98%; *R*–(adj) 82.87%; R^2(pred) 22.73%.

molasses, 10 ml inoculum size, and 0.65 g/l, KH_2PO_4. The lowest production was expressed in experiment number 8. A second-order polynomial function was fitted at the PHB yield as follows:

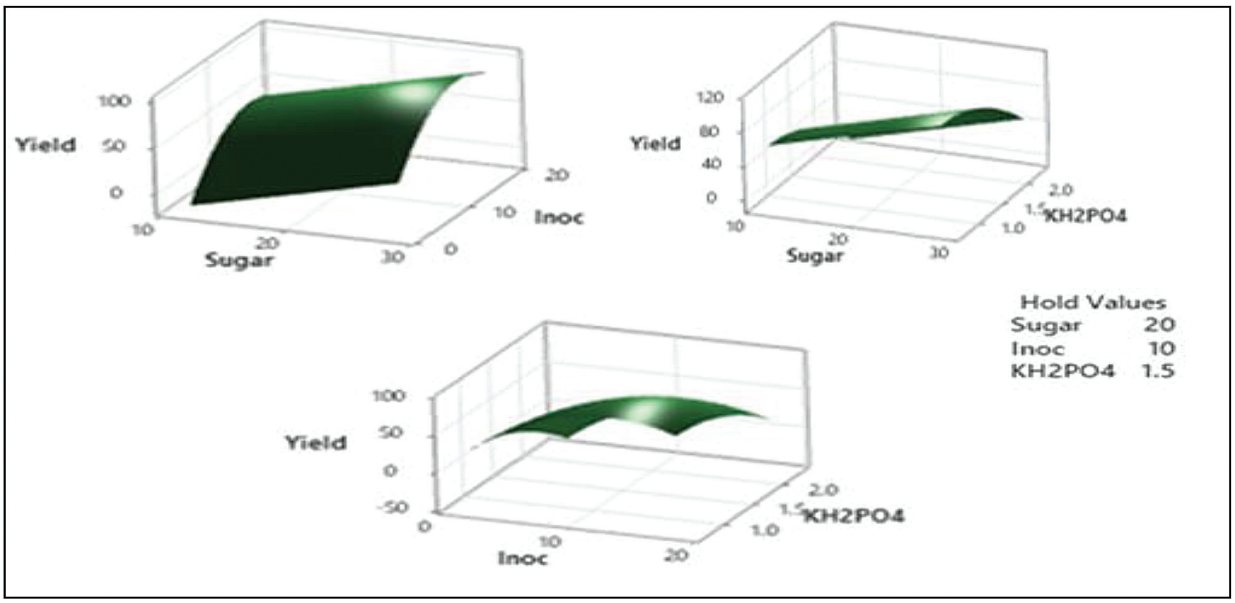
$$Y \text{ yield} = -152.1 + 9.41\ x_1 + 16.91\ x_2 + 45.5\ x_3 - 0.180\ x_1^2 - 0.526\ x_2^2 - 0.650 x_3^2 - 0.009\ x_1x_2 + 0.56\ x_1x_3 - 2.06\ x_2x_3.$$

Where *y* represents the PHB concentration and x_1, x_2, and x_3 are the sugarcane, inoculum size, and KH_2PO_4 coded values, respectively. The statistical significance of the equation was verified by the *F* test, and the ANOVA for the response surface quadratic model is shown in Table 3. All of the linear terms as well as x_2^2 (inoculum size square) had a significant effect ($P<0.005$), whereas the other square terms had insignificant effect. Nevertheless, all the interactive effects had insignificant effects on PHB accumulation. The results of the ANOVA of the regression model demonstrated the significance of the model as an evident from the high *F* values and the low *P* values. The lack of fit (0.003) with PHB productivity also indicated that the results obtained were well-fitting with the model.

The regression equation presented a determination coefficient R^2=0.9098%. Thus, this model can explain ~90.98% of the variability in the dependent variables; 9.02% was affected by other variables. The R^2 value is always between 0 and 1. The model is more powerful if the R^2 is close to 1.0. The R^2(adj) and R^2 were 82.87 and 90.98%, respectively, for PHB yield, which better predicted the response. These values indicate a high degree of correlation between the experimental and predicted value (22.73%). The optimal values of the different factors vary depending on the microorganisms used to make PHB and their isolation location, altering their physiological features [4,43,44].

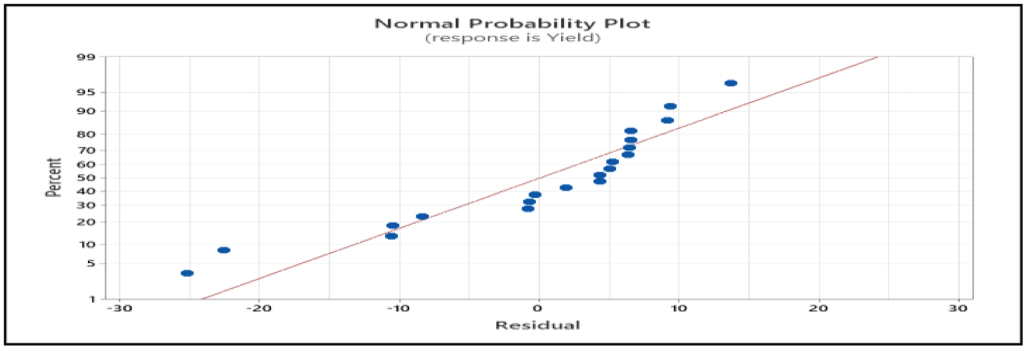
The three-dimensional response surface has been plotted on the basis of the conducted model equation to investigate the interaction among the variable (Fig. 9). The normal probability plot (Fig. 10) indicated that the effects that lie along the

Figure 9



Three-dimensional response surface showing the effect of sugarcane concentration, inoculum size, and KH_2PO_4 on PHB production. PHB, polyhydroxybutyrate.

Figure 10



Residual plot.

normal probability line are negligible, whereas the significant effects are those far points.

Conclusion

A novel bacterial isolate capable of producing PHB was obtained from clay soil in El-Menia, Upper Egypt, and was identified using 16S rRNA gene sequencing as *B. safensis* (NR_113945.1). Plackett–Burman design and RSM approach through central composite sequential design were applied for optimizing PHB production. Sugarcane molasses was the best carbon source. ^{1}H-NMR and Fourier transform infrared spectroscopy analysis demonstrated the structure of the PHB produced. Hence, the present study provided useful data for the industrial production of PHB using a safe local isolate with a cheap carbon source.

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Conflicts of interest

There are no conflicts of interest.

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