Article

Production and optimisation of Polyhydroxyalkanoates (PHA) from *Bacillus subtilis* using response surface methodology

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Abstract

Polyhydroxyalkanoates (PHAs) are storage materials, accumulated by various bacteria as energy and carbon reserve materials. They are biodegradable and biocompatible; hence, they can be used in packaging and carrier molecules in the agricultural field. In the present study, we aim to produce PHA by isolating and screening PHA-accumulating bacteria by performing primary and secondary screening with Sudan Black B dye and Nile Blue A, respectively. Out of 110 isolates, 19 isolates showed bluish black colouration. The quantification of PHA was carried out by cell dry weight, and biofilm was obtained by the sodium hypochlorite-chloroform method. The isolate K2(2) showed maximum PHA production and was optimised for PHA production using the software RSM. The isolate was further classified up to the genus level by studying its morphological and biochemical characteristics, as well as 16s rRNA sequencing, and it was found to be Bacillus subtilis. The extracted PHA polymer was characterised by Fourier Transform Infrared (FTIR) spectroscopy. The isolate was tested on various parameters to check its effect on PHA production. The isolates were good candidates for the industrial production of PHA.

Keywords PHA; bioplastic; biodegradable; *Bacillus subtilis*; RSM modeling.

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1 Introduction

Plastics are utilised in almost every industry, ranging from the automobile to food and medicines. Plastics have acquired significance since the 1940s and have replaced glass, wood, and metal in packaging applications. Plastic materials used for packaging purposes are being discarded into landfills and marine environments. The accumulated plastic in landfills cannot be disposed of by burning (Behera et al., 2022). The rapid increase in production and consumption of plastics has led to serious plastic waste problems. To overcome this problem, eco-friendly bioplastics, which are durable, environmentally degradable, recyclable, and produced from renewable resources, can replace synthetic plastics (Bhuwal, 2013; Chanprateep, 2010). PHAs have gained

more importance due to their close analogy to plastic, which is a well-known biopolymer that belongs to the family of naturally occurring bio-polyesters synthesised by various recombinant bacteria, transgenic plants and microorganisms, including certain fungi, archaea and bacteria (*Bacillus megaterium, Azotobacter beijerinckii, Ralstoniaeutropha, Alcaligenes latus, and Pseudomonas oleovorans)*(Getachew et al., 2016). The accumulation of PHA by microorganisms can be stimulated under unbalanced growth conditions when nutrients such as nitrogen, phosphorus, or sulphate become limiting, when oxygen concentration is low, or when the C: N ratio of the feed substrate is higher. These biopolymers are stored intracellular in the form of inclusion bodiesand may accumulate up to 80-90% of dry cell weight (Chanprateep, 2010). During starvation, PHA serves as a carbon and energy source and is rapidly oxidised by retarding the degradation of cellular components, combating the adverse conditions in rhizospheric soil. The significant characteristics of PHA, such as its biodegradability, biocompatibility, chemical diversity, and its manufacture from renewable carbon resources, have led to its widespread commercial and research interest (Dash et al., 2014). The composition of a PHA molecule usually comprises 600 to 35,000 (R)-hydroxy fatty acid monomer units. Based on the number of carbon atoms present in a PHA monomer, it is classified into three categories (short-chain length PHA, medium-chain length PHA, long-chain length PHA) (Burdon et al., 1942). PHA has a wide range of potential applications because of its desired features, such as biocompatibility, biodegradability, and negligible cell cytotoxicity. Hence, the potential application of PHA is in various fields involving packaging, medical, and coating materials (Hamdy et al., 2022). PHA has a distinct advantage in the medical field over silicone, a traditionally used polymer, which is believed to have maligned effects as a target drug delivery. Because of the high cost of the raw materials required in the production process, PHA manufacture is quite expensive. Utilising alternate carbon sources (natural products, industrial wastes, and agro-industrial residues) for manufacturing through different fermentation processes is one of the methods that have been employed to lower this cost (Chaudhry et al., 2011). RSM is a powerful technique that may be used to optimise media components and other crucial factors that affect the synthesis of biomolecules (Du et al., 2001).

2 Methodology

2.1 Isolation of PHA-producing microorganisms

Samples were collected from the Pinnara dumpsite, UdhnaMethi Khadi, Kharvasa Khadi, and segregated waste soil. Before serial dilution, 1 g of soil was suspended in 10 mL of sterile distilled water. Sterile minimal salt medium (MSM) agar plates were used to plate the aliquots. The plates were incubated for 24 to 48 h at 30°C. To sustain viability, the resulting microbial colonies were purified, kept at 4°C on nutrient agar slants, and subcultured every 15 days (Getachew and Woldesenbet, 2016).

2.2 Primary screening

- **Plate assay method:** The isolates were cultured for 24 h at 30°C after being restreaked on MSM agar medium with 1% glucose as the sole carbon source and 0.05% ethanolic solution following incubation. Sudan black B was applied to the colonies and left for half an hour. The extra stain was removed from the colonies by washing with 96% ethanol (Chaudhry et al., 2011; Kalaivani and Sukumaran, 2013).
- **Colony fluorescence Method:** All the isolates were restreaked on MSM agar medium with 1% glucose, having nile blue A dye, incubated for 24 h at 30°C, and observed under UV for fluorescence (Liu et al., 1998).
- **Sudan black B staining:** All the isolates were qualitatively tested for PHA production using Sudan black B dye. After 48 h of incubation on MSM agar medium, the heat-fixed smear was stained with Sudan black for 10 mins, then rinsed in xylene, and counter-stained with safranin for 10 seconds (Du et al., 2001; Hazer and Steinbüchel, 2007; Madison and Huisman, 1999).

2.3 Secondary screening, production, and extraction of PHA

All the PHA producers obtained from primary screening were subjected to secondary screening under liquid conditions. 100 ml simplified MSM medium, having glucose and glycerol as carbon sources and inorganic nitrogen, with inoculum (1%) for 48 h and 72 h, enhanced the growth of cells and PHA accumulation. Sodium hypochlorite and the hot chloroform technique were used to extract PHA. The mixture was centrifuged for 20 minutes at 10,000 rpm following incubation. After cleaning with distilled water, the resultant cell pellet was placed in a screw cap tube that had been previously weighed and over-dried at 60°C in an oven. The air-dried cell weight (CDW) remained constant (Dash et al., 2014; Hamdy et al., 2022). The pellet was resuspended with 4% sodium hypochlorite (10 ml), incubated in a shaker for 30 mins, and centrifuged at 10,000 rpm for 10 mins. The supernatant was discarded, and the pellet was washed with distilled water, acetone, and methanol. Double volume of hot chloroform was added to the tube and kept in a shaker for 24 h. The extract was poured into a pre-weighed screw cap tube and observed for film formation. The chloroform was evaporated, and the PHA film was obtained (Du et al., 2001; Getachew and Woldesenbet, 2016; Hazer and Steinbüchel, 2007; Kalaivani& Sukumaran, 2013). The highest accumulation of PHA in bacteria was obtained and selected for further optimisation of the parameters by the following formula:

PHA Accumulation (%) = (Weight of extracted PHA/Cell dry weight) * 100.

2.4 Preliminary characterisation and molecular identification of PHA-producing bacteria

The selected bacterial isolates were characterised based on its growth characteristics, Gram's staining and biochemical tests (Indole production test, glucose phosphate broth test, Voges–Proskauer test, citrate utilization test, carbohydrate fermentation test, gelatin liquefaction test, hydrogen sulfide (H_2S) production test, and urea hydrolysis test)(Hamdy, 2022). The 16S rRNA sequencing was conducted at Gene Explorer Diagnostics and Research Centre Pvt. Ltd.

2.5 Optimisation of PHA using OFAT

It was conducted in an MSM medium. The aim was to enhance both PHA production and the growth rates of the selected isolates. Key parameters such as temperature, pH, incubation time, carbon source, and nitrogen source were carefully adjusted and monitored to achieve optimal conditions for PHA production (Madison et al, 1999).

2.5.1 Effect of waste carbon sources on PHA production

100ml of sterile MSM was prepared in different conical flasks and supplemented with different 1% waste carbon sources such as sugarcane bagasse, corncob, orange peel, orange pulp, papaya peel, cottonseed oil, peanut oil, industrial waste oil, and waste engine oil. All flasks were inoculated with 3 MFU inoculum and incubated for 72 h at 37°C. PHA production was estimated after 72 h using the cell dry weight method (Du et al, 2016; Chaudhry, 2013).

2.5.2 Effect of carbon percentage on PHA production

All flasks were inoculated with selected carbon sources at different percentages (0.5, 1, 1.5, 2) and incubated for 72 h at 37°C. PHA production was estimated after 72 h using the cell dry weight method (Du et al, 2016; Chaudhry, 2013).

2.5.3 Effect of nitrogen source on PHA production

100 ml of sterile MSM containing the optimal carbon source was prepared in different conical flasks and supplemented with different 1% nitrogen sources (urea, peptone, ammonium chloride, ammonium sulphate, potassium nitrate), incubated at 37°C in a shaking incubator. PHA production was estimated after 72 h using the cell dry weight method (Salim et al., 2014).

2.5.4 Effect of nitrogen percentage on PHA production

All flasks were inoculated with selected nitrogen sources at different percentages (0.5, 1, 1.5, 2) and incubated for 72 h at 37°C. PHA production was estimated after 72 h using the cell dry weight method (Salim et al, 2014).

2.5.6 Effect of different pH on PHA production

100 ml of sterile MSM supplemented with the best carbon and nitrogen source was inoculated at different pH levels (5, 6, 7, 8, 9). After incubation, PHA production was estimated after 72 h using the cell dry weight method (Hamdy et al, 2022).

2.5.7 Effect of different temperatures on PHA production

100 ml of sterile MSM supplemented with the best carbon, nitrogen, and pH at different temperatures (20ºC, 30ºC, 40ºC, 50ºC). After incubation for 72 h, PHA production was estimated using the cell dry weight method (Hamdy et al, 2022).

2.6 Process parameters optimisation by RSM

The levels of the significant parameters and the interaction effects between various medium constituents, which may influence the PHA production significantly, were analysed and optimised by response surface central composite design (CCD) (Hamdy et al, 2022). The concentrations of three major components, carbon%, nitrogen%, and inoculum size, were optimised, keeping the temperature, pH, and incubation time constant throughout the experiments. Each factor in the design was studied at five different levels (−α, −1, 0, +1, +α) using Design Expert version 6.0 (Stat-Ease Inc., Minneapolis, MN, USA) statistical software. The isolate produced PHA after being optimised with the Design Expert version 12 software from Stat-Ease Inc. in Minneapolis, USA. A total of 20 experimental trials with six replicates of the centre point were recommended by the CCD. The concentration of peanut oil (A), ammonium sulphate (B), and inoculum (C) at five distinct levels—−α (−1.68), low (-1), medium (0), high (+ 1), and + α(+ 1.68)—were used to optimise PHA production using the CCD model. To evaluate whether the polynomial expression could statistically predict the observed values, an analysis of variance (ANOVA) was conducted to compare the predicted and measured data. They are predicated on how the concentration of the two main components functions while maintaining the same values for the other variables. The 2D contour and 3D response surface plots are frequently used to graphically depict the regression equation. Surface plot contours can reveal varied interactions (Hamdy et al, 2022).

2.7 Polymer analysis

The chemical structure and the functional groups of the extracted PHAs were analysed using FTIR spectroscopy.

3 Results and Discussion

3.1 Isolation of PHA-producing microorganisms

A total of 110 isolates were obtained, of which 55 were obtained from Pinnara dumpsite, 19 from segregated waste soil, 19 from UdhnaMethi Khadi, and 17 from Kharvasa Khadi.

3.2 Primary screening

PHA producers showed bluish-black colonies due to the lipid nature of PHA granules, and showed fluorescence under UV light using Nile Blue A. Out of 110 isolates, 19 were found to accumulate PHA. Screening of PHA producers was also performed by microscopic analysis of PHA granules present in various isolates stained with Sudan black B dye (Fig. 1).

3.3 Secondary screening, production, and extraction of PHA

Among all the isolates, K2(2), isolated from UdhanaMethi Khadi, was found to be the highest PHA producer with 50% of PHA content. All the other isolates were also found to be considerable PHA producers. Hence, K2(2), which was found to be gram-positive bacilli, was selected for further studies (Fig. 2, Table 2).

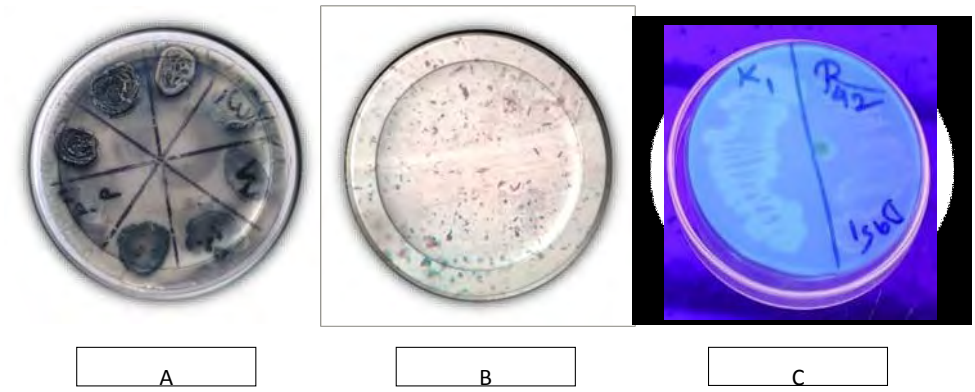

Fig. 1 The images of the Sudan black plate assay method (A), staining method (B), and Nile Blue A (C).

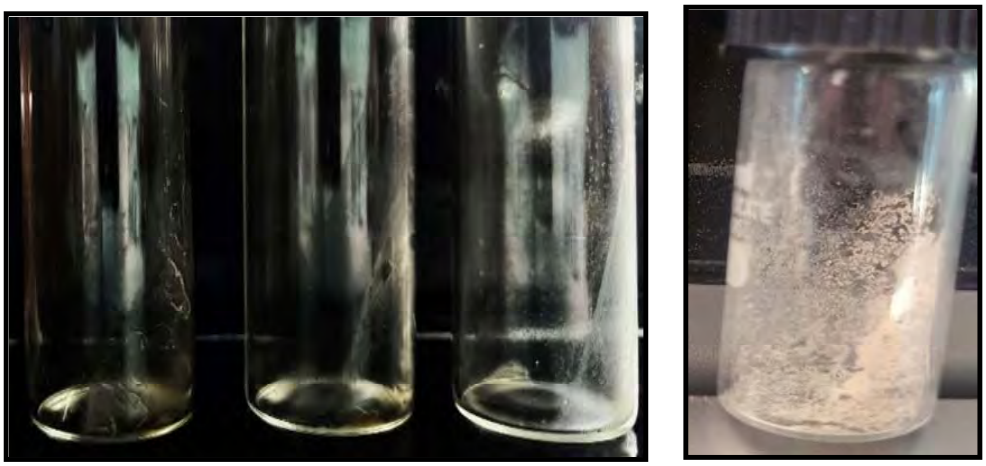

Fig. 2 PHA film formation and extracted PHA powder.

Table 2 PHA content produced by various isolates using glucose and glycerol as a carbon source.

Sample	Glucose		Glycerol	
	48 h % PHA content	72 h % PHA content	48 h % PHA content	72 h % PHA content
K2(2)	16	41	60	50
D9(6)	25	40	5	5
K2(2)	50	50	5	5
D9(8)	2.32	1.81	nil	5
D5(11)	5	15	9	9
D5(12)	5	5	10	nil
P5(3)	2	16	33	33
D4(3)	10	Nil	nil	nil
D9(7-1)	20	20	10	20
D9(4)	nil	Nil	66	66

W1(1)	10	10	nil	nil
D9(1)	5	Nil	5	nil
D9(3)	10	Nil	10	nil
P5(1)	nil	Nil	nil	nil
K1(1)	nil	Nil	nil	nil
W(3)	nil	Nil	nil	nil
D9(7-2)	15	10	nil	50
D9(7-3)	nil	Nil	nil	nil

3.4 Preliminary characterization and molecular identification of PHA-producing bacteria

The K2(2) isolate was the highest producer of PHA and was analyzed to the genus level with biochemical tests and with the sequencing of 16s rRNA. Positive results were obtained for the VP test, citrate utilisation test, and gelatin liquefaction test. In contrast, negative results were obtained for indole, MR test, H_2S production test, CO_2 production test, and urea hydrolysis test. For the TSI test, the slant and butt were both alkaline. The organism that produced PHA (K2(2)) had the morphological, physiological, and biochemical traits of the *Bacillus* genus. The isolated bacteria were rod-shaped, aerobic, and Gram-positive. On nutritional agar, colonies are pale yellow with uniform edges. The PHA-producing isolate (K2(2)) was identified using the 16s rRNA gene. Based on the alignment results, the 16s rRNA sequences of the selected strain showed a 100% similarity to *Bacillus subtilis*.

The sequence was submitted to NCBI with the accession number **PV789674.1.**

3.5 Optimisation of process parameters

3.5.1 Effect of waste carbon sources on PHA production

Among the various carbon sources tested to evaluate their effect on PHA yield, corncob was found to be the best carbon source with 53% of PHA content. The reason that corncob is rich in polysaccharides is that it can be easily converted into fermentable sugars, which can directly enter into the metabolism, and it can easily get involved in PHA synthesis (Fig. 3).

3.5.2 Effect of carbon percentage on PHA production

The corncob was found to be the best carbon source. The result depicted an increase in PHA yield relative to increased glycerol availability up to a certain concentration, after which it tends to drop. The isolate accumulated the highest polymer at a carbon concentration of 1.5% in the production medium with 50% PHA content (Fig. 4).

3.5.3 Effect of nitrogen source on PHA production

The nitrogen source is important for the growth of the cell biomass; its effect was checked on PHA production. Among the various nitrogen sources, Ammonium Sulphate was the best nitrogen source for PHA production, with 49% of PHA content. The increased C: N ratio, resulting from the low nitrogen content in ammonium sulphate, may have contributed to enhanced PHA production (Fig. 5).

3.5.4 Effect of nitrogen percentage on PHA production

When Ammonium sulphate was used as the nitrogen source, PHA production was 49%. An increase in Ammonium sulphate concentration up to 1.5 % led to a 51% enhancement in PHA yield (Fig. 6).

3.5.5 Effect of different pH on PHA production

Tests were conducted at various pH values (5, 6, 7, 8, and 9). The selected strain produced the highest PHA production, 1500mg/ml, at pH 8. The selected isolates only produced small amounts of PHA at pH 7 and pH 9 (Fig. 7).

3.5.6 Effect of different temperatures on PHA production

The effect of different incubation temperatures (20 ° C, 30 ° C, 40 ° C, and 50 ° C) on PHA accumulation was evaluated. The highest PHA yield, 52.17%, was observed at 40 ° C (Fig. 8).

The study of the combined effect of all the optimised parameters on PHA production was carried out to check the yield of PHA. The media with 1.5% corncob as carbon source, ammonium sulphate as nitrogen source, having a pH of 8, was inoculated with 10% inoculum size and incubated at 40°C for 72 h at 120 rpm. The figure shows a nearly 3 times increase in cell biomass as well as PHA. Both the PHA content relative to cell biomass was found to be increased by 78% (Fig. 9).

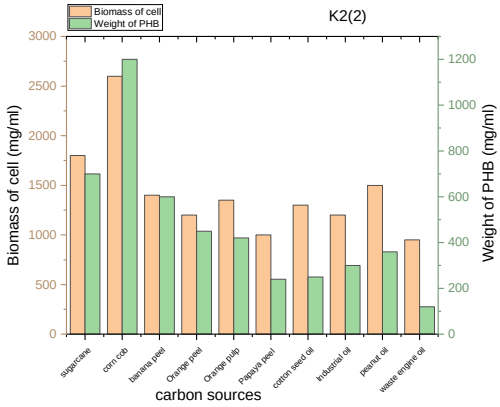

Fig. 3 Effect of various carbon sources.

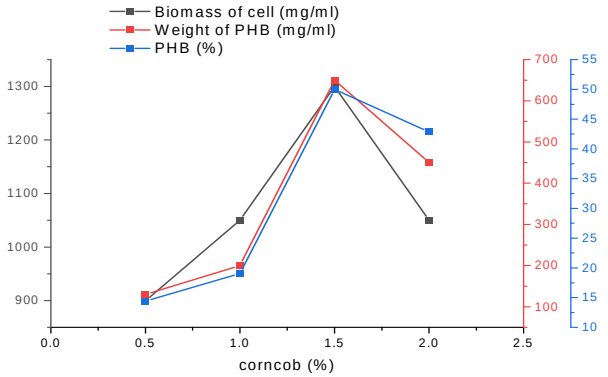

Fig. 4 Effect of carbon concentration.

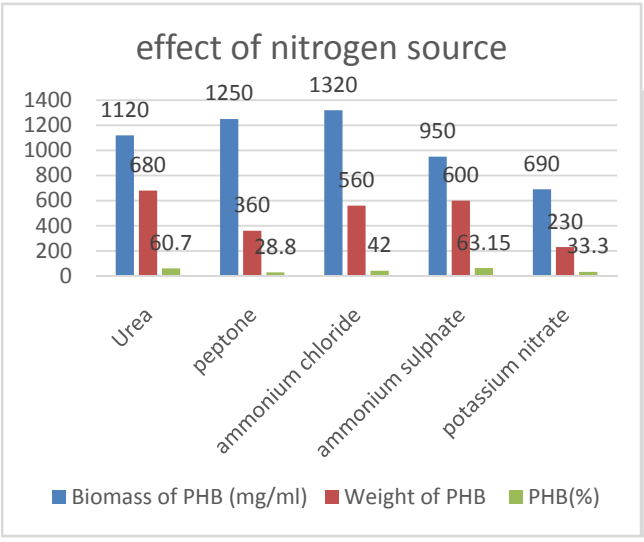

Fig. 5 Effect of various nitrogen sources.

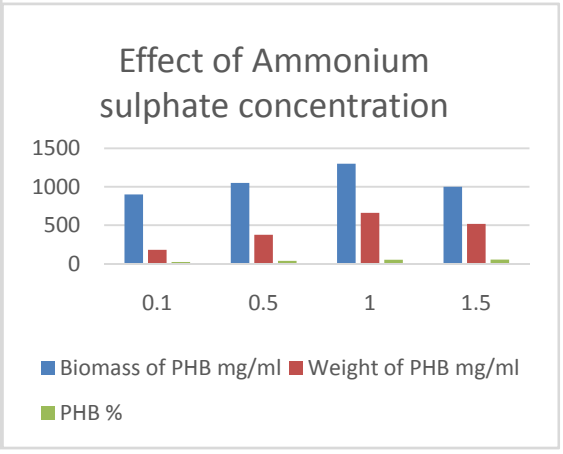

Fig. 6 Effect of nitrogen concentration.

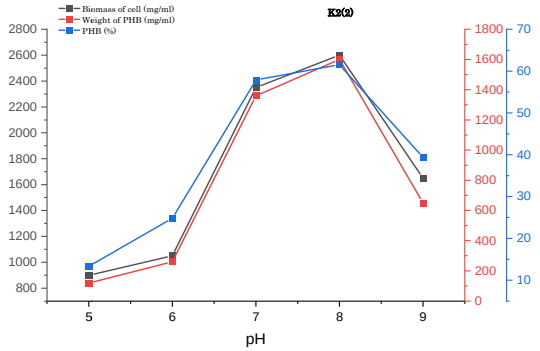

Fig. 7 Effect of pH.

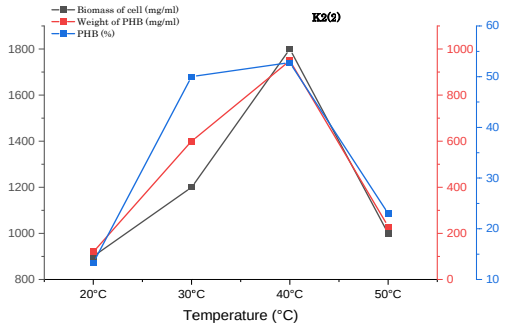

Fig. 8 Effect of temperature.

Fig. 9 PHA film obtained after the optimised cycle.

3.6 Statistical optimisation of PHA production

Table 2 shows the test variables used for CCD.

Table 2 Test variables and levels of CCD to optimise carbon concentration, ammonium sulphate concentration, and inoculum size for PHA production.

	Name	Units	Low	High	-alpha	+alpha
A [Numeric]	corncob	g/l	10	30	3.18207	36.8179
B [Numeric]	ammonium sulphate	g/l	5	20	-0.113446	25.1134
C [Numeric]	inoculum size	ml	1	10	-2.06807	13.0681

Table 3 shows the Response 1 (PHA mg/L) attained based on the results.

Table 3 Combination of the experiment based on CCD for optimisation of corncob concentration, ammonium sulphate concentration, and inoculum size in the production of PHA.

Std	Run	Factor 1 (A: corncob) %	Factor 2 (B: ammonium sulphate) %	Factor 3 (C: inoculum size) %	Response 1 (PHA) %
18	1	20	12.5	5.5	26.5
11	2	20	0.113446	5.5	12.2
6	3	30	5	10	17.2
19	4	20	12.5	5.5	29.6
8	5	30	20	10	22.5
2	6	30	5	1	15.8
9	7	3.1820716949257	12.5	5.5	1.56
20	8	20	12.5	5.5	28.6
7	9	10	20	10	15.6
14	10	20	12.5	7.5	33.2
4	11	30	20	1	20.1
13	12	20	12.5	2.06807	29.5
17	13	20	12.5	5.5	30.3
5	14	10	5	10	1.2
1	15	10	5	1	0.8
15	16	20	12.5	5.5	28.9
12	17	20	25.113446228806	5.5	20.2
10	18	36.817928305074	12.5	5.5	19.6
3	19	10	20	1	18.6
16	20	20	12.5	5.5	30.2

ANOVA was employed to assess whether the polynomial model could reliably predict the experimental outcomes. The regression equation was illustrated using contour plots, which depict the interaction between two key variables while keeping the remaining factors constant. The ANOVA for response 1 (PHA content) quadratic model was tabulated. The Model F-value of 10.50 implies the model is significant. There is only a 0.05% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case, A, A^2, B^2, and C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model (Table 3, 4).

Table 4 ANOVA for Quadratic model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1792.66	9	199.18	39.13	< 0.0001	significant
A-corncob	366.00	1	366.00	71.90	< 0.0001	
B-Ammonium sulphate	46.73	1	46.73	9.18	0.0127	
C-inoculum size	1.35	1	1.35	0.2659	0.6173	
AB	19.84	1	19.84	3.90	0.0766	
AC	14.58	1	14.58	2.86	0.1214	

BC	120.12	1	120.12	23.60	0.0007	
A^2	752.72	1	752.72	147.87	< 0.0001	
B^2	400.27	1	400.27	78.63	< 0.0001	
C^2	5.85	1	5.85	1.15	0.3089	
Residual	50.91	10	5.09			
Lack of Fit	29.33	5	5.87	1.36	0.3721	not significant
Pure Error	21.57	5	4.31			
Cor Total	1843.57	19				

Figs 10-12 shows the 3D contour graphs. The contours of the surface plots can be used to infer the interaction between the variables under investigation (Figs 10, 11, 12). Tables 5 and 6 show the Lack of Fit F-value of 2.92 implies the Lack of Fit is not significant relative to the pure error. There is a 13.24% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good if we want our model to be fit. The **Predicted R^2** of 0.8586 is in reasonable agreement with the **Adjusted R^2** of 0.9475; i.e. the difference is less than 0.2. **Adeq Precision** measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Your ratio of 18.735 indicates an adequate signal. This model can be used to navigate the design. The actual (experimental) PHA production reactions and the projected ones were almost the same. The anticipated values for PHA content were calculated. Using regression analysis and contrasted with experimental data, showing that the predicted and actual response values agreed with the suggested solution for the constraint was peanut concentration (1%), ammonium sulphate concentration (1.5%), and Inoculum (1.25%) to maximise PHA content (1.75g/L) (Table 5, 6).

Table 5 Response 1: PHA content.

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0445	0.0398	0.2727	0.1523	
2FI	0.8388	0.0250	0.1592	-0.6021	
Quadratic	**0.0001**	**0.6951**	**0.8503**	**0.7007**	**Suggested**
Cubic	0.5973	0.5487	0.8330	0.0424	**Aliased**

Table 6 Fit statistics.

Std. Dev.	2.47	**R^2**	0.9685
Mean	20.11	**Adjusted R^2**	0.9402
C.V. %	12.28	**Predicted R^2**	0.7839
		Adeq Precision	16.1749

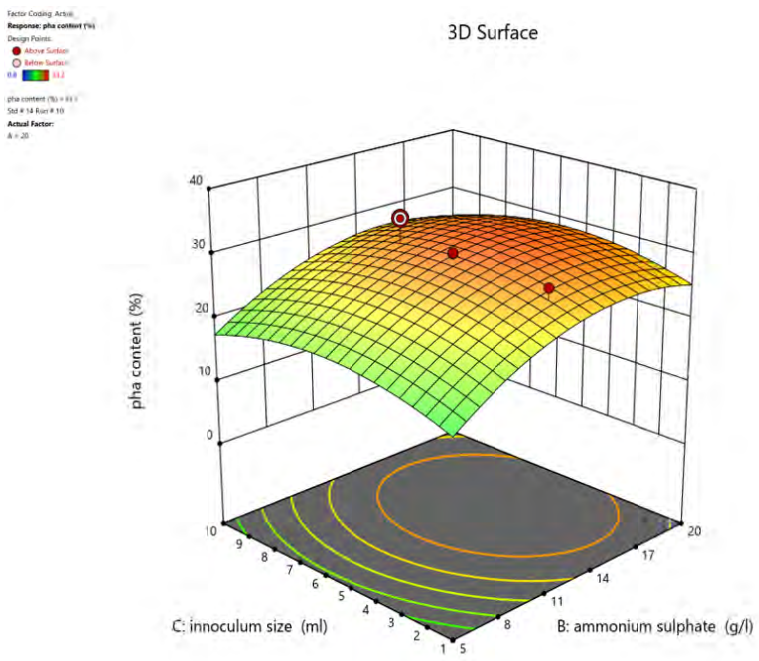

Fig. 10 Effect of inoculum size concerning ammonium sulphate on PHA.

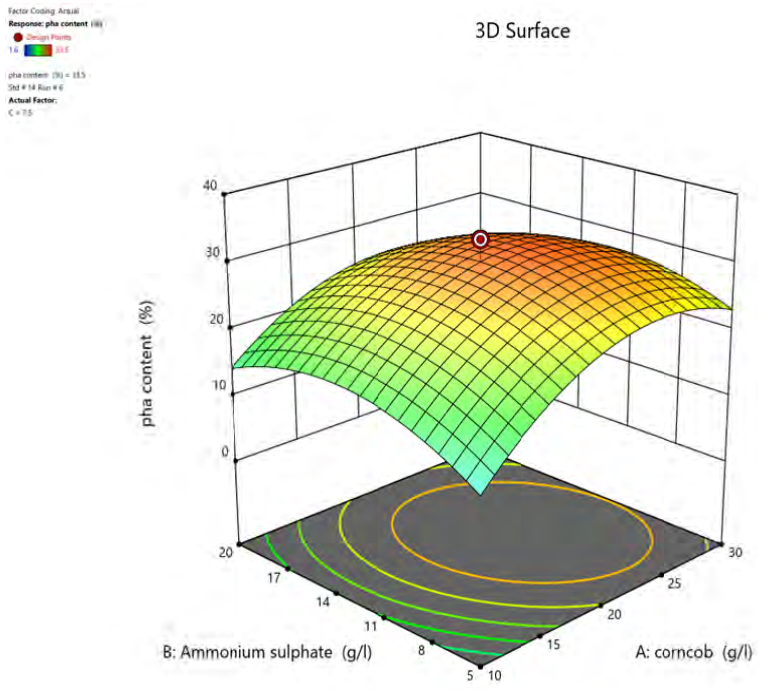

Fig. 11 Effect of ammonium sulphate concerning corncob on PHA.

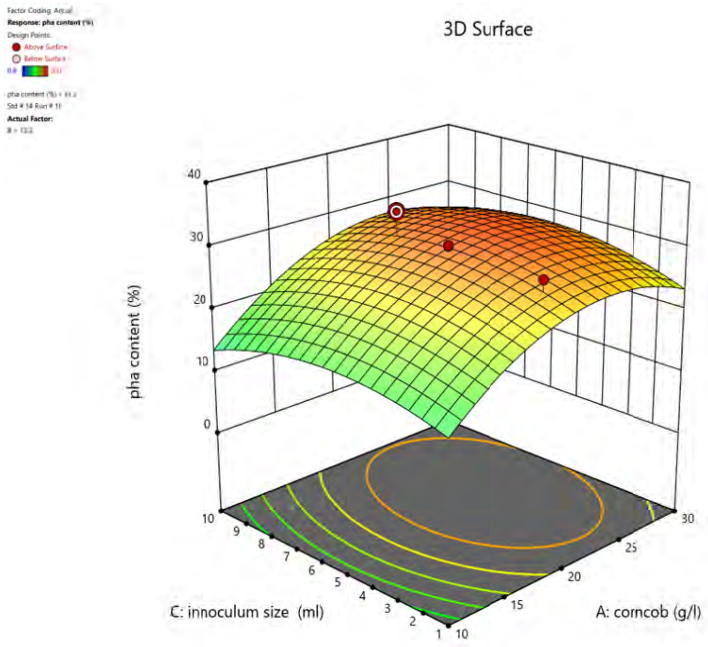

Fig. 12 Effect of inoculum size concerning the Corncob on PHA.

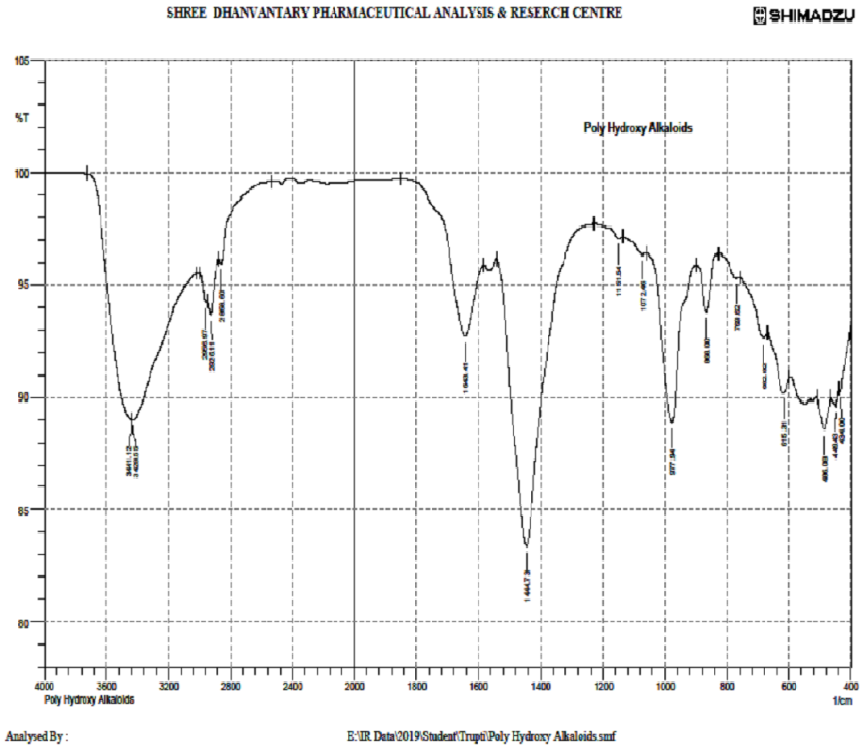

Fig. 13 IR spectrum of the PHA produced by the selected isolate no. K2(2).

3.7 Polymer analysis

A prominent band that emerged at 1636.30 cm^{-1} is ascribed to the carbonyl (C=O) stretching of the ester group. The band represents the asymmetrical deformation of the C-H bond in CH2 groups at 1453 cm^{-1}, whereas CH_2S groups are represented by the band at 1457.38 cm^{-1}. The band at 3436 cm^{-1} was formed by the terminal OH groups, confirming that the polymer is PHA (Fig. 13).

4 Conclusion

In this study, *Bacillus subtilis* was successfully isolated from industrially contaminated soil and identified as a high-yield PHA producer. Among 110 isolates, K2(2) demonstrated the highest PHA production capacity (up to 50% under standard conditions), which increased to 78% under optimised conditions. This reflects the organism's strong potential for bioplastic production. Corncob emerged as the most effective carbon source, yielding 57% PHA. This may be due to the presence of glycerol and fatty acids that are easily assimilated into the PHA biosynthesis pathway. These findings align with Salmiati et al (2014) report of 51% PHA per dry mass production using corncob and support the notion that oils are favourable substrates for PHA synthesis due to their high carbon content and energy efficiency. Ammonium sulphate was the most efficient nitrogen source, yielding 54% PHA. Its low nitrogen content likely enhanced the C: N ratio, a critical factor in triggering PHA accumulation. Similar observations were made by Getachew and Woldesenbet (2016), who reported 63.1% PHA with ammonium sulphate at pH 8 and 37°C, reinforcing the reliability of our results. Optimal conditions (1.5% corncob, 1.5% ammonium sulphate, pH 8, 40°C) were determined using OFAT and further validated and refined using RSM. The statistical model was found to be highly significant ($p < 0.0005$), with a strong correlation coefficient ($R^2 = 0.9212$) and non-significant lack of fit ($p = 0.13$), indicating a robust prediction model. Adeq Precision value (7.948) confirmed the signal-to-noise ratio was adequate for model navigation. FTIR analysis confirmed the presence of characteristic PHA functional groups, including a carbonyl ester peak at 1636 cm^1 and CH_2 deformation bands, affirming the polymer's identity. These findings validate the biochemical and statistical approach used in the study. In conclusion, *Bacillus subtilis* isolated from UdhnaMethi Khadi is a promising candidate for sustainable PHA production. The use of low-cost, agro-industrial waste substrates like corncob and statistical optimisation techniques such as RSM can significantly improve yield, making the process economically viable for industrial applications. Future studies should focus on pilot-scale fermentation, purification cost reduction, and evaluating the mechanical properties of the produced bioplastic for commercial use.

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