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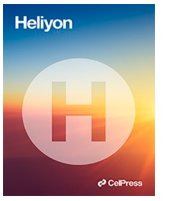

Research article

Optimizing Polyhydroxyalkanoate production using a novel *Bacillus paranthracis* isolate: A response surface methodology approach

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ABSTRACT

Microorganisms have emerged as promising resources for producing economical and sustainable bioproducts like Polyhydroxyalkanoate (PHA), a biodegradable polymer that can replace synthetic plastics. In this study, we screened a novel isolate, *Bacillus paranthracis* RSKS-3 strain, to produce PHA from sewage water, identifying it using Whole Genome Sequence. This study represents the first report on optimizing PHA production using B. paranthracis RSKS-3, employing Design Expert 12.0 software. Our findings reveal that four factors (temperature, inoculum size, potassium dihydrogen phosphate, and magnesium sulfate) significantly affect PHA production in the Plackett-Burman design experiment. Through Response Surface Methodology, we optimized PHA production to 0.647 g/L with specific values for potassium dihydrogen phosphate (0.55 %), inoculum size (3 %), magnesium sulfate (0.055 %), and a temperature of 35 °C, in agreement with the predicted value of 0.630 g/L. This optimization resulted in a substantial 13.29-fold increase in PHA production from 0.34 g/L to 4.52 g/L, underscoring the promising role of *B. paranthracis* RSKS-3 in eco-friendly PHA production and advancing sustainable bioproduct development.

1. Background Introduction

The increasing need for cost-effective and environmentally-friendly substitutes for synthetic plastics has generated considerable attention towards microorganisms as possible reservoirs for bioproducts, particularly Polyhydroxyalkanoate (PHA), a biodegradable polymer. The primary objective of this investigation is to examine the application of *Bacillus paranthracis* RSKS-3, a strain that has been carefully selected due to its capacity to synthesize polyhydroxyalkanoates (PHA) from sewage water. This technique represents a unique and innovative contribution to the existing body of knowledge in this research domain. One notable component of this study is the utilization of statistical optimization techniques to produce PHA, facilitated by the implementation of Design Expert 12.0 software. This pioneering effort represents a novel approach in the field of sustainable bioproduct development, which holds significant promise for future applications. The results, specifically obtained using the Plackett-Bruman design experiment and response surface approach, reveal the significant parameters that have an impact on the production of PHA. These factors have been found to contribute to a substantial increase in yield, reaching a 13.29-fold improvement when operating under optimized conditions. In summary, this

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research not only highlights the potential of *B. paranthracis* RSKS-3 in environmentally friendly PHA synthesis but also signifies a notable advancement in the development of sustainable options within the field of biodegradable polymers.

2. Background

Plastics have become an indispensable part of daily human life, with their widespread use in various industrial sectors resulting in millions of tons of plastic waste generated annually [1–3]. Unfortunately, the amount of plastic waste produced exceeds its production, and by 2050, the Geneva Environment Network estimates that more than 33 billion tons of plastic waste will accumulate on Earth due to the exponential growth of the human population [4,5]. To address this issue, there is a growing interest worldwide in using bio-based polymers as an environmentally friendly alternative to conventional petrochemical-based plastics with similar physicochemical properties to meet the increasing demands of the rapidly growing biomedical and industrial sectors. Bioplastic offers biodegradability, non-toxic breakdown, a smaller carbon footprint, and a renewable source than typical plastics [6–9]. These characteristics make bioplastics like PHA more sustainable and advantageous for the environment [10–12]. Polyhydroxyalkanoates (PHAs) are a type of natural biodegradable polyester produced by microorganisms and are considered an environmentally viable alternative to commercial synthetic plastics [13–15].

PHA accumulation and bioprocess optimization is critical for achieving the desired amount and quality of biopolymer production by wild-type strains. Two-step growth techniques have been employed by various authors, such as Sabapathy et al. (2019) and Umesh et al. (2022), where the first stage involves the generation of bacterial biomass to achieve the highest density and modify the producer's development circumstances, followed by a nutrient reduction in the second phase, which aims to encourage PHA accumulation [16,17]. To optimize the medium for commercial application of any metabolite, various physicochemical factors of the medium's composition, such as carbon, nitrogen, macro and micro-minerals, pH, temperature, aeration, and inoculum age, must be considered [18]. One way of enhancing optimization processes is through statistical approaches that use statistical methods like Response Surface Methodology and Design of Experiments (DoE). Under DoE, for efficient tools, Bayesian tools, Simulated annealing, and Genetic algorithms are used for the optimization of expensive functions, escaping local optima, and diverse solutions, respectively [19].

Statistical experimental design, such as factorial design and response surface approach (RSM), can reduce the hindrance of the OFT (One Factor at a Time) protocol, save time and cost, and investigate the relatively large number of factor interactions. By selecting the most important influencing factors and analyzing their interactions, factorial design and response surface approach can be used to show their combined influence (RSM) [20,21]. To the best of our knowledge, this is the first study on its own that represents the initial investigation into using a synthetic propagation medium containing minimal nutrients to enhance the production of PHA by *B. paranthracis* RSKS-3. The optimization of PHA production characteristics of the isolate was carried out through statistical methods, with glucose serving as the sole carbon source in the process. The Plackett-Burman design and Response Surface Methodology was identified as the most prevalent approach for determining the factor most likely to enhance PHA production. The importance of PB design is to efficiently screen multiple factors using a minimal number of experiments and RSM to efficiently provide optimized conditions for maximum PHA production. We also formulated a hypothesis to test for a PB design for PHA production by *B. paranthracis* RSKS-3:

H(0): Optimization factors do not significantly enhance PHA production by *B. paranthracis* RSKS-3.

H(1): Optimization factors significantly enhance PHA production by *B. paranthracis* RSKS-3.

Based on the hypothesis testing, the significant factors screened from PB design were optimized using RSM to enhance PHA production.

3. Materials and methodology

3.1. Culture identification and maintenance

B. paranthracis RSKS-3 was isolated from sewage samples collected from Budha Nala, Maheru India (31.2527354 Latitude, 75.6824303 Longitude). It is a gram-positive, motile, and spore-forming rod, and was identified using 16s rRNA assay. The isolates were preserved in glycerol stock for further study. For preservation, a 50 % (v/v) glycerol solution was prepared and *B. paranthracis* RSKS-3 colonies from the nutrient agar plate were inoculated to a 2 mL Eppendorf having 1.5 mL 50 % glycerol for preservation at −80 °C temperature [22].

3.2. Initial PHA production medium

PHA production was done using a conventional liquid broth minimal medium containing glucose (10 g/L), $(NH_4)_2SO_4$ (3 g/L), KH_2PO_4 (3.18 g/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L), $ZnSO_4{\cdot}7H_2O$ (0.02 g/L), Na_2HPO_4 (3.8 g/L), peptone (1.5 g/L). The chemicals were purchased from the Loba Chemie Pvt Ltd., Mumbai, Maharastra, India A production medium of 1 L was prepared in an Erlenmeyer flask and autoclaved at 121 °C with 15 psi for 15–20 min. Also, the production process was conducted under physiological conditions at an initial pH of 7.0. An inoculum size of 10^8 cells/mL (*B. paranthracis* RSKS-3) was inoculated under aseptic conditions and the flask was kept at a temperature of 37 °C with continuous agitation at 150 rpm for 48 h [22,23]. The product was recovered as explained in 2.4.

3.3. Statistical experimental design for optimization process

3.3.1. Plackett-Burman design: screening of significant factors

The Plackett-Burman design (PBD) is highly efficient method within the broader framework of Design of Experiments (DOE), also known as Statistical Experiment Design (SED) [24]. It is valuable for screening numerous variables to identify the most significant factors influencing PHA production by Bacillus paranthracis RSKS-3. P.B. is designed for two-level factorial screening that has two levels: high and low levels [25] facilitating the estimation of direct effects with minimal experiments.

In this study, PHA production impacting 11 variables was selected using the PB design. The experimental trials were conducted at a 95 % relative significance level. PBD was used to code the independent variables' fundamental values into High (+1) and Low (−1) levels, which were then presented in Table 1 [12,26].

Biopolymer synthesis was conducted in a 100 mL Erlenmeyer flask. The flask contained a working volume of 50 mL of a minimal medium whose composition was determined using Design Expert 12.0 software (Table 1). The entire experiment in the flask was performed in triplicate for each step. The product was extracted as explained in 2.4.

3.3.2. Box Behnken Design (BBD): response surface methodology for optimization

Three significant factors or variables (KH_2PO_4, inoculum size, $MgSO_4$, and temperature) influenced PHA production by B. paranthracis RSKS-3 was used in BBD as a part of response surface methodology (RSM).

BBD consists of four factors at 3 levels (−1, 0, +1) and produced 30 experiments using Design Expert 12.0 to fulfill a second-order polynomial model. The general quadratic equation that represents this model is:

$$\mathrm{PHA} = \beta 0 + \beta 1(\mathrm{A}) + \beta 2\ (\mathrm{B}) + \beta 3\ (\mathrm{C}) + \beta 4\ (\mathrm{D}) + \beta_{11}\ (\mathrm{A})^2 + \beta_{22}\ (\mathrm{B})^2 + \beta_{33}\ (\mathrm{C})^2 + \beta_{44}\ (\mathrm{D})2 + \beta_{12}\ (\mathrm{A})(\mathrm{B}) + \beta_{13}\ (\mathrm{A})(\mathrm{C}) + \beta_{14}\ (\mathrm{A})(\mathrm{D}) + \beta_{23}\ (\mathrm{B})(\mathrm{C}) + \beta_{24}\ (\mathrm{B})(\mathrm{D}) + \beta_{34}\ (\mathrm{C})(\mathrm{D}) + \epsilon$$

In this equation, PHA = predicted Polyhydroxyalkanoate production.

A = KH_2PO_4; B = inoculum size; C= $MgSO_4$; D = temperature are the respective factors

β0,βi, βii, and βij denotes the model coefficients that quantify the linear, quadratic, and interaction effects of the factors on PHA production

ϵ = accounts for the residual error term in the model.

The quadratic equation will be used to predict PHA production under combinations of factors determined by the Box-Behnken model. The design of BBD with factors and their levels were represented in Table 2 [27,28].

Again, the PHA production was conducted in a 100 mL Erlenmeyer flask with a working volume of 50 mL of a minimal medium with composition and conditions: glucose (10 g/L), $(NH_4)_2SO_4$ (3 g/L), $ZnSO_4{\cdot}7H_2O$ (0.02 g/L), Na_2HPO_4 (3.8 g/L), peptone (1.5 g/L). However, KH_2PO_4, inoculum size, $MgSO_4$, and temperature were determined using Design Expert 12.0 software (Table 2). The entire experiment in the flask was performed in triplicate for each step. The product was extracted as explained in 2.4.

3.3.3. Statistical analysis

In the PBD, statistical tools including ANOVA and regression analysis based on Pareto charts were used to pinpoint significant factors affecting PHA production. Furthermore, Box-Cox transformation and Prediction Error of Sum of Squares (PRESS) analysis were performed to ensure the model's accuracy and reliability.

During the subsequent BBD, ANOVA and regression analyses were utilized to evaluate the model's fit, focusing on the significance of the F-value. Additionally, the interaction of factors was examined through 2-D contour plots, which provided a visual depiction of the interaction effects and their impact on PHA production.

3.4. Extraction and recovery of PHA

The cells were pelleted through centrifugation at 8000×*g* for 10 min to purge out the supernatant. After that, one mL of distilled water was mixed with the cells in Eppendorf tubes. The tubes were centrifuged again at 10,000×*g*, and the supernatant was discarded. The pellets were dried at 60 °C until reached a consistent weight. Next, PHA extraction was performed on pelleted cells. The pellets

Table 1
Plackett-Burman design was used to select various experimental factor levels for *B. paranthracis* RSKS-3 to produce PHA.

Sr. no.	Factors	Low level (-1)	High level (+1)
1	Glucose	2 g/L	10 g/L
2	pH	5	8
3	Temperature	30 °C	40 °C
4	Incubation period	24 h	96 h
5	Inoculum size	1 %	5 %
6	KH_2PO_4	0.1 %	1 %
7	Na_2HPO_4	0.1 %	1 %
8	$MgSO_4$	0.01 %	0.1 %
9	$ZnSO_4$	0.001 %	0.01 %

Table 2
Box-Behnken design was used to select various experimental factor levels for *B. paranthracis* RSKS-3 to produce PHA.

Sr. no.	Factors	Low level (-1)		High level (+1)
1	KH_2PO_4	0.1 %	0.55 %	1 %
2	Inoculum size	1	3	5
3	$MgSO_4$	0.01 %	0.055 %	0.1 %
4	Temperature	30 °C	35	40 °C

were treated with a sodium hypochlorite solution to dissolve cellular debris and placed in an orbital shaker incubator at 30 °C for 2 h. After incubation, the tubes were centrifuged at 8000×*g* for 20 min, and the supernatant was removed. The pellets were then rinsed with distilled water, and the resulting liquid was transferred to pre-weighed Eppendorf tubes. The tubes were centrifuged at 10,000×*g* for 20 min, and the supernatant was discarded. The pellets were washed with acetone and methanol to eliminate any remaining cell debris and then dried at 60 °C until they reached a constant weight [29,30]. Later the granules were dissolved in hot chloroform and dispensed into watch glass to evaporate the solvent [31].

3.5. PHA identification using UV spectroscopy

After dissolving 1 mg of dried PHA granules in 2 mL of concentrated sulphuric acid, the mixture was boiled for 10 min. UV spectra were then measured in the 800 to 200 nm range, utilizing a calibration baseline of sulphuric acid and standard crotonic acid obtained from Central Drug House Laboratory Reagent in New Delhi, India [22,23].

3.6. 2.6 PHA characterization

PerkinElmer equipment, FT-IR spectroscopy was used to analyse the functional groups present in both the extracted and control PHA material. The examination focused on wavelengths ranging from 4000 to 400 cm^{-1}. XRD spectroscopy was utilized to analyse the crystalline structure of the acquired Polyhydroxyalkanoate (PHA) particles. The device utilized a radiation wavelength of 1.5405 Å to scan a range from 1 to 70° in 2θ. The isolated PHA was analyzed for morphology using a scanning electron microscope (FE-SEM: JEOLJSM-7610F PlusEDS: OXFORD EDS LN2 free). The investigation of the structure and composition of PHA was conducted using NMR spectroscopy at 600 MHz. Finally, the thermal stability of the samples was assessed using a Mettler TG50 thermobalance. 5 mg of dried samples were measured on the device and analyzed under a nitrogen flow rate of 20 mL/min. The temperature was increased to 500 °C at a pace of 10 °C per minute [22,23].

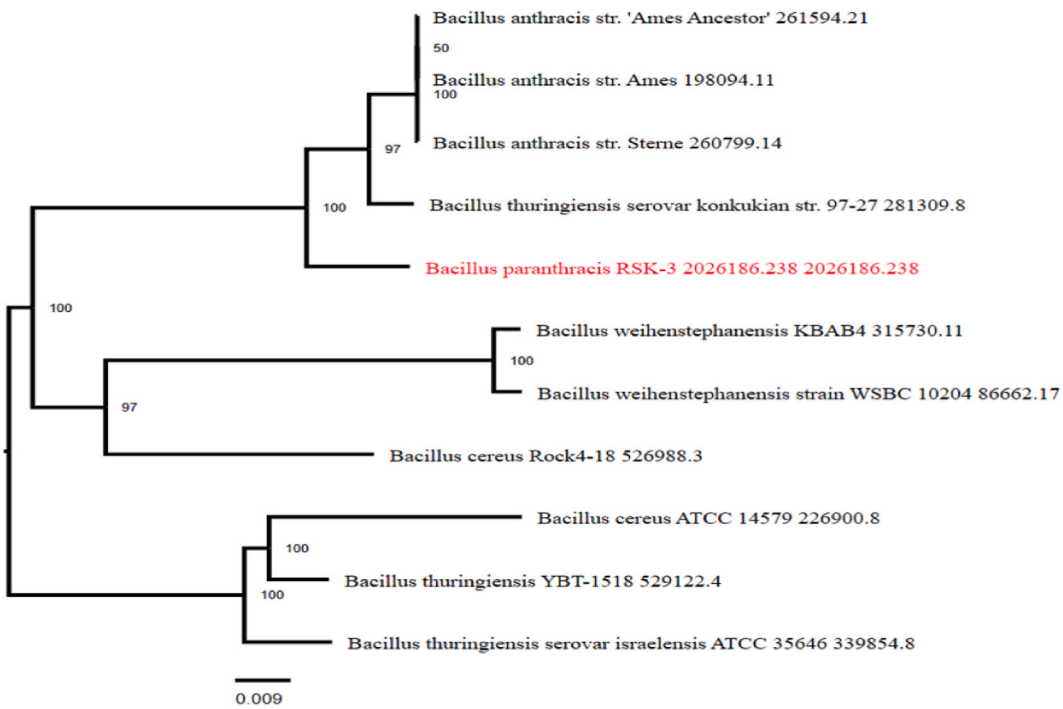

Fig. 1. Phylogenetic analysis based on 16s rRNA of RSKS-3 (*B. paranthracis* RSKS-3) having SAMN39897631 accession number in NCBI database.

4. Result and discussion

4.1. Culture characteristics and identification

B. paranthracis RSKS-3 was isolated from the sewage water from Budha Nala, Maheru, India (31.2527354 Latitude, 75.6824303 Longitude). The bacterium was characterized using Next Generation Sequence platform, which also analyzed 16s rRNA through which a phylogenetic tree was constructed employing the maximum likelihood statistical method and 400 replicas were made using a bootstrap test as shown in Fig. 1. The accession number OR506140.1 has been assigned to the novel *B. paranthracis* RSKS-3 strain, which has been formally placed in the NCBI database as bio-sample no. SAMN39897631.

4.2. Statistical experimental design for screening and optimization of PHA production

4.2.1. Plackett-Burman design

One factor at time (OFAT) tests provide insights into individual factor effects, they may not capture potential interactions between factors, which might be examined further using a factorial design or optimization techniques in tools, Design of tests (DOE) software such as Design-Expert. Design of Experiments (DOE) is a statistical methodology used to systematically plan, conduct, and analyse experiments to understand the relationship between input factors (independent variables) and output responses (dependent variables). DOE provides a framework for optimizing a process or system by identifying the key factors that influence the response and determining the optimal settings for these factors.

The basic concept of DOE is to design and execute a set of experiments where the factors of interest are varied systematically, and the responses are measured under controlled conditions. This enables the experimenter to isolate and quantify the effects of each factor on the response and to identify the most important factors that affect the response.

DOE can be used to identify the optimum settings of input factors to achieve the desired output responses, and to investigate interactions between factors that may affect the response. It is widely used in industries such as manufacturing, engineering, and pharmaceuticals to improve product quality, increase process efficiency, and reduce costs [32,33].

DOE involves several steps, including:

- Defining the problem and setting the objectives of the experiment.
- Selecting the factors to be studied and the range of values to be tested.
- Designing the experiment by selecting an appropriate experimental design, such as a factorial design, response surface design, or mixture design.
- Experimenting and collecting the data.
- Analysing the data using statistical methods to estimate the effects of the factors and identify the optimal settings.

Concluding and making recommendations based on the results of the experiment.

DOE is a powerful tool for identifying the key factors that affect a process or system and optimizing its performance. It enables the experimenter to make informed decisions based on objective data and to achieve significant improvements in quality, efficiency, and cost-effectiveness.

Experimental trials comprising 12 runs were conducted using Design Expert 12.0 and investigated consecutively to identify the significant variables contributing to PHA formation by B. paranthracis RSKS-3 isolated from the sewage water of Buddha Nala from Lovely Professional University, India The primary goal of this screening study was to determine the relative importance of eleven parameters (glucose, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}7H_2O$, Na_2HPO_4, $MgSO_4$, pH, temperature, inoculum size) at two levels (low and high) on PHA yield, depicted in milligrams per mL. A relative significance threshold of 95 % was maintained, and twelve shake flask experiments with three center points were performed. A student t-test was involved to calculate the significance of nine factors. Table 3 presents the experimental and projected PHA values.

Statistical analysis of PHA generation (mg/L) for the P.B. design is displayed as an ANOVA table in Table 4. Furthermore, Fig. 2 shows a Pareto chart illustrating the impact of variables on PHA production.

An analysis of variance was performed on the experimental design, revealing that the model is significant with an F-value of 69.21. The probability of noise causing such a high F-value is only 1.43 %. The model's significance is indicated by probability $> F >$ b values of 0.0500, and based on the P-value, the significant model terms can be determined:

B- temperature (p-value = 0.0184),

D-inoculum size (p-value = 0.0153), D- KH_2PO_4 (p-value = 0.0024), and.

H– $MgSO_4$ (p-value = 0.0196).

A Pareto chart is a graphical method used to display the relative importance of different factors in a Plackett-Burman (PB) design. It is a bar chart where the factors are ordered from the most significant to the least significant, based on their effect on the response variable. In Fig. 2, the Pareto chart of nine factors for PHA yield showed that four factors (KH_2PO_4, inoculum size, temperature, and $MgSO_4$) were above the t-value limit of 4.30. These factors were identified to have the largest effect on the PHA response. The height of each bar in the chart represents the magnitude of the effect of each factor, and the bars are arranged in descending order of magnitude.

The Pareto chart can help to identify the most significant factors affecting the response variable and to prioritize which factors

Table 3
Plackett-Burman design's experimental model on PHA yield by *B. paranthracis* RSKS-3.

Run	pH	Temperature (°C)	Glucose (%)	Inoculum size (%)	Incubation period (hrs)	KH_2PO_4 (%)	Na_2HPO_4 (%)	$MgSO_4$ (%)	$ZnSO_4$ (%)	PHA concentration (mg/mL)	
										Predicted values ± 2.17 SD	Experimental values
1	+1	−1	+1	−1	+1	+1	−1	−1	−1	69.66	70 ± 1.5
2	+1	−1	−1	+1	−1	+1	−1	+1	+1	81.2	80 ± 2.05
3	+1	+1	+1	−1	−1	−1	+1	+1	−1	44.2	43 ± 1.25
4	+1	+1	−1	+1	+1	−1	−1	+1	−1	48.6	50 ± 1.67
5	−1	−1	−1	−1	+1	−1	+1	+1	+1	54.6	55 ± 2.35
6	+1	+1	−1	−1	+1	+1	+1	−1	+1	58.2	58 ± 1.76
7	−1	+1	+1	−1	−1	+1	−1	+1	+1	68.6	70 ± 2.09
8	−1	+1	−1	+1	−1	+1	+1	−1	−1	74.6	75 ± 1.87
9	+1	−1	+1	+1	−1	−1	+1	−1	+1	52.6	54 ± 2.89
10	−1	−1	−1	−1	−1	−1	−1	−1	−1	40.2	40 ± 0.92
11	−1	−1	+1	+1	+1	1	1	0.1	0.001	100.2	100 ± 1.22
12	−1	+1	+1	+1	+1	0.1	0.1	0.01	0.01	44.2	43 ± 1.76

Table 4
ANOVA and Regression analysis of PB design on nine factors for PHA yield.

Source	S.S.	df	MSE	F-value	p-value	Main impact	Standardized error	
Model source	8.82	9	0.9805	69.21	0.0143			**Significant**
A-pH	0.1633	1	0.1633	11.53	0.0769	−0.1167	0.034	
B-Temperature	0.7500	1	0.7500	52.94	0.0184	−0.2500	0.034	
C–C-source	0.1008	1	0.1008	7.12	0.1165	0.0917	0.034	
D-Inoculum size	0.9075	1	0.9075	64.06	0.0153	0.2750	0.034	
E-Incubation period	0.0408	1	0.0408	2.88	0.2317	0.0583	0.034	
F–KH_2PO_4	5.88	1	5.88	415.06	0.0024	0.7000	0.034	
G-Na_2HPO_4	0.2133	1	0.2133	15.06	0.0604	0.1333	0.034	
H–$MgSO_4$	0.7008	1	0.7008	49.47	0.0196	0.2417	0.034	
J-$ZnSO_4$	0.0675	1	0.0675	4.76	0.1607	−0.0750	0.034	
Residual	0.0283	2	0.0142					
Cor Total	8.85	11						

df = degree of freedom, SS = sum of squares, MSE = Mean square error.
R^2 = 99.68 %; $R^2_{adjusted}$ = 98.24 %; $R^2_{predicted}$ = 88.48 %.

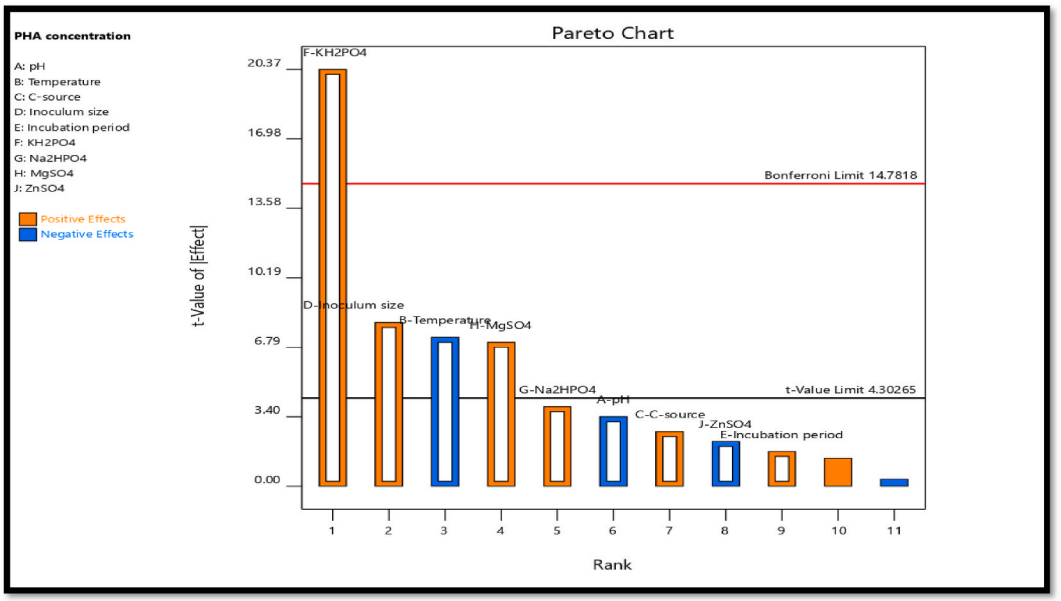

Fig. 2. Pareto chart of standardized effect of fermentative parameters for PHA produced by *B. paranthracis* RSKS-3. Four factors (KH_2PO_4, inoculum size, temperature, and $MgSO_4$) out of nine shows significant effect as they crossed above t-value limit.

should be further investigated or optimized. It can also help to communicate the results of the PB design clearly and concisely.

The predicted vs. experimental plot can help to assess the validity and accuracy of the PB design model. The predicted values are calculated based on the PB design model, while the experimental values are obtained from the experimental data. If the PB design model is accurate and valid, then the points on the plot should fall along a straight line with a slope of 1.0. In Fig. 3, the data of 12 runs for nine factors falls along the straight line which indicates the data is accurate and does not have any outliers.

However, any deviation from this straight line may indicate that the PB design model is not accurate or that there are other factors affecting the response variable that were not accounted for in the PB design. The predicted vs experimental plot can also help to identify any outliers or other patterns in the data that may affect the validity of the PB design model. Outliers are data points that deviate significantly from the overall pattern of the data and may be indicative of errors or unusual circumstances in the experiment. By evaluating the predicted vs experimental plot, researchers can determine whether the PB design model is valid and reliable and whether any modifications or adjustments to the model are necessary. This can help to ensure that the results of the PB design are accurate and can be used to improve the process or system being studied.

Box-Cox transformation is another statistical technique that is often used to transform data to satisfy the assumptions of normality and equal variance that are required by many statistical methods. The Box-Cox transformation involves applying a power transformation to the data, which can help to reduce the impact of outliers and other sources of variability. In the context of a Plackett-Burman (PB) design, perturbation and Box-Cox transformation can be used together to improve the robustness and reliability of the results. After conducting the initial PB design, the researcher may decide to perturb the system by changing the levels of the factors, as discussed in the previous answer. If the response variable is not normally distributed or does not have equal variance, then the

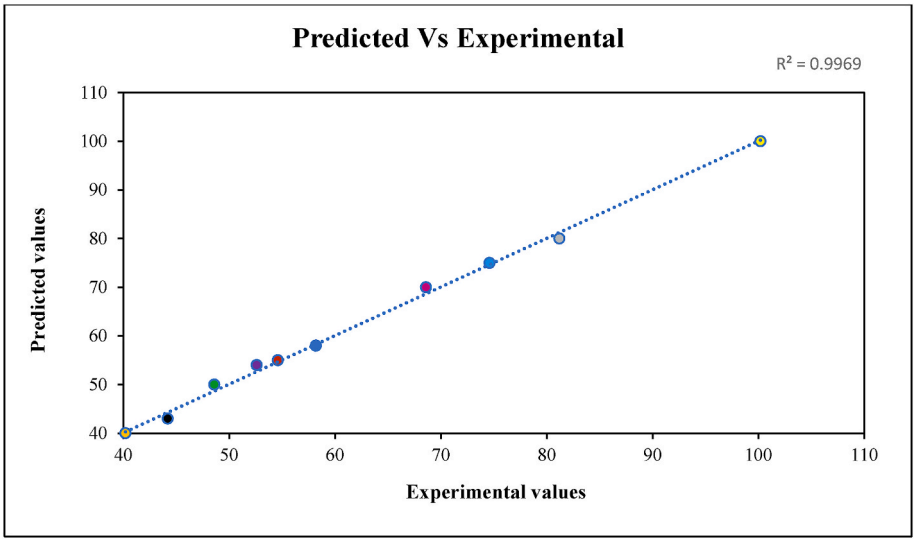

Fig. 3. The predicted Vs experimental values PHA concentration of Plackett-Burman design for 12 runs of nine factors.

results of the PB design may be affected by this non-normality or heteroscedasticity. To address this issue, the researcher can apply a Box-Cox transformation to the response variable, which can help to normalize the data and reduce the impact of outliers. The parameter lambda (λ) in a Box-Cox plot dictates the kind and strength of the transformation used to the data, and the plot assists in determining the ideal lambda value for obtaining normalcy in the converted data. However, in Fig. 4, the box-cox plot does not require any transformation as the data or values from the PB design of nine factors are normalized data.

The main objective of the experimental screening design was to examine the variables that significantly impact the production of

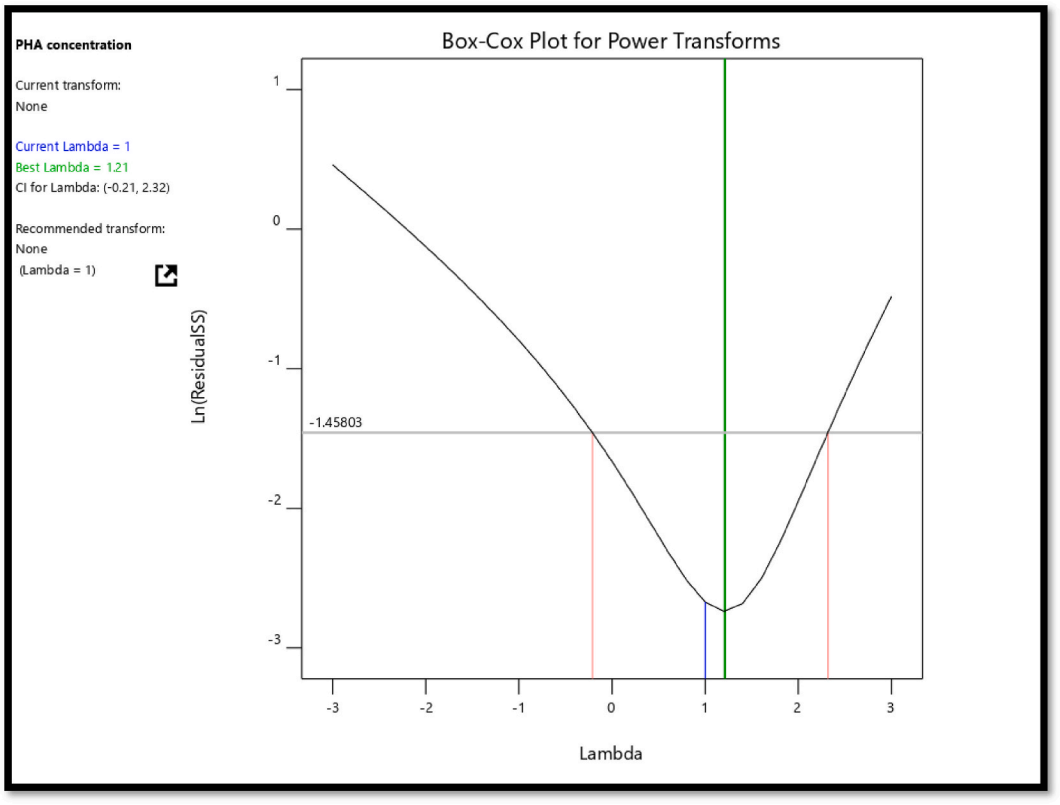

Fig. 4. Box-Cox plot of PB design for nine factors. The Box-cox plot here represents the data is normalized and do not require any transformation.

PHA. Plackett-Burman studies were conducted to provide an initial idea of which production factors might be influential before conducting a more specific screening. Experimental variables were selected based on their close relationship to the production in previous shake flask cultures. The Pareto chart provided a clear visual representation of the standardized effect of each variable on production. In conclusion, the concentrations of KH_2PO_4, $MgSO_4$, temperature, and inoculum size affected PHA formation due to growth. The study revealed that temperature and inoculum size played a significant role in increasing PHA yield. However, the substantial role of KH_2PO_4 and $MgSO_4$ in PHA yield indicates the crucial importance of K and Mg ions in different transport systems and cytoplasmic signaling. However, Potassium (K) and magnesium (Mg) ions are essential in bacterial cells, especially in relation to polyhydroxyalkanoate (PHA) synthesis. Potassium ions operate as crucial cofactors for enzymes that are vital for metabolic pathways linked to PHA production, guaranteeing the best enzymatic performance during precursor metabolism and polymerization processes. K ions have a role in osmoregulation by maintaining turgor pressure and aiding in nutrient transport, which is crucial for PHA synthesis. Magnesium ions are essential for DNA synthesis and stability, contributing to the transcription and translation processes required for gene expression related to PHA synthesis. Magnesium ions serve as cofactors for different enzymes, activating essential biosynthetic processes such as precursor formation and polymerization. Additionally, magnesium ions play a role in energy transfer activities and assist in stabilizing cellular structures, hence impacting the energetic equilibrium necessary for PHA production. Both potassium (K) and magnesium (Mg) ions are essential for the complex cellular mechanisms that contribute to the effective synthesis of PHAs in bacterial cells.

Regression formula for the model's PHA in mg/L:

3.46111 - (0.077778 x pH) - (0.050000 x **Temp.**) + (0.022917 x **C-source**) + (0.137500 x **Inoculum size**) + (0.001620 x **Incubation period**) + (1.55556 x **KH_2PO_4**) + (0.296296 x **Na_2HPO_4**) + (5.37037 x **$MgSO_4$**) - (16.66667 x **$ZnSO_4$**)

The PRESS (Prediction Error of Sum of Squares) was calculated and noted as 1.11. PRESS is a method of assessing the predictive performance of a statistical model by measuring the difference between the predicted values and the experimental values of a response variable for each observation in a dataset. The PRESS statistic is calculated by fitting a model to all the observations except one and then using the fitted model to predict the response value for the omitted observation. This process is repeated for each observation in the dataset, and the sum of the squared differences between the predicted and experimental values is calculated. The PRESS statistic is useful in evaluating the predictive performance of a model, especially when the model is used for prediction on new, independent data. By evaluating the model on the same dataset that was used for model fitting, the PRESS statistic provides an estimate of the model's prediction error that is less biased than the traditional R-squared or mean-squared error statistics. It can be used to compare the predictive performance of different models or to select the best subset of predictors in a model selection procedure. However, it should be noted that the PRESS statistic is sensitive to the sample size and the number of predictors and should be used in conjunction with other diagnostic measures to fully assess model performance [34].

The resultant KH_2PO_4, $MgSO_4$, temperature, and inoculum size as significant variables in the Plackett-Burman (PB) design for Polyhydroxyalkanoate (PHA) production can be attributed to their essential biological functions in microbial growth, metabolism, and the PHA biosynthesis process. The phosphorus derived from KH_2PO_4 is required to synthesize DNA and RNA. This element plays a critical function in upholding the structural integrity of genetic material and cell membranes [35]. Moreover, the involvement of phosphorus molecules, such as adenosine triphosphate (ATP), is of utmost importance in facilitating the intracellular transfer of energy. This process plays a critical role in supporting the energy-demanding production of polyhydroxyalkanoates (PHA) [36]. Magnesium ions (Mg^{2+}), which are supplied by $MgSO_4$, play a crucial role as indispensable cofactors for a multitude of enzymes that participate in fundamental cellular mechanisms, including DNA replication, transcription, and translation. In addition, magnesium plays a crucial role in the stabilization of ribosome structures, which are essential for the process of protein synthesis, a fundamental component of Polyhydroxyalkanoate (PHA) formation [37].

In addition, temperature plays a crucial role as it has a direct impact on the rates of microbial growth and metabolic activity. The effectiveness of PHA production can be influenced by the temperature range at which microbial cultures are maintained. The phenomenon under consideration exerts an influence on the catalytic efficiency of enzymes, the pace at which substrates are absorbed, and the overall metabolic processes that are essential for the synthesis of polyhydroxyalkanoates (PHA) [38]. The size of the inoculum, however, plays a critical role in the initiation and sustenance of a robust microbial culture. The initial cell density plays a crucial role in determining the future pace at which PHA-producing bacteria can convert the available substrates into PHA. Ensuring an optimum quantity of inoculum is crucial in establishing a resilient culture capable of effectively conducting Polyhydroxyalkanoate (PHA) production.

The incorporation of KH_2PO_4, $MgSO_4$, temperature, and inoculum size as significant variables in PB design is indicative of their essential contributions to the proliferation, metabolic activities, and production of PHA in microbial cells. The optimization of PHA synthesis in microbial cultures is contingent upon several crucial parameters, which play a pivotal role in facilitating the effective utilization of resources and fostering a sustainable output of PHA.

Hence, it is clear from Fig. 1 that 4 factors significantly affected the PHA production by *B. paranthracis* RSKS-3 and reject the null hypothesis that stated no factors studied in PB design have a significant effect on PHA production and accept the alternate hypothesis that stated at least one factor studied in PB design has a significant effect on PHA production by *B. parathracis* RSKS-3. However, there were limitations like more micronutrients as well as external factors were not considered in this study. These limitations can be overcome in future studies related to PHA production by *B. paranthracis* RSKS-3.

4.2.2. Box-Behnken Design and response surface methodology

For the PHA optimization study, a Box-Behnken design using glucose as the carbon source was designed. The four significant factors A, B, C, and D (KH_2PO_4, inoculum size, temperature, and $MgSO_4$, respectively) through analysis from PB design were used in different combinations to influence PHA synthesis by *B. paranthracis* RSKS-3 under BBD. The BBD used four factors on three levels (−1, 0, +1) that produced 30 experiments to fit a model of a second-order polynomial. The study was to comprehend how these factors affect or influence PHA production by *B. paranthracis* RSKS-3. Tables 5 and 6 represent factors and levels for BBD, and analysis of ANOVA for PHA with three variables. The regression analysis based on linear models (A, B, C, and D) showed the significance of the factors relatively. Similarly, quadratic models (A^2, B^2, C^2, and D^2) and two-way interaction models (AB, AC, AD, BC, BD, CD) were also found to be significant. The following equation shows a second-order polynomial equation for maximum PHA production that includes linear, square, and 2-way interaction of the factors:

$$\text{PHA concentration}=(-0.99)+0.44*A+0.088*B+0.051*C+4.77*D+0.013*A*B-0.004*A*C-0.47*A*D-0.002*B*C-0.04*C*D-0.082*A^2-0.0009*B^2-0.0003*C^2-10.61*D^2$$

4.2.2.1. Fit of model. The predicted and experimental values for response (PHA production, g/L) are represented in the design matrix in Table 5. The maximum PHA g/L was 0.781 g/L (Table 5). The competency between the BBD model and the evaluation of fitness was carried out by ANOVA along with the statistical significance of the model. The statistical significance analysis was analyzed based on the *F* test and *P* test (Table 6). The model *F* values of 30.07 (Table 6) indicate the model significance and the lack fit value of 1.50 for PHA was found to be less than the *F* values. This confirms the model's significance and helps in predicting the overall PHA yield.

4.2.2.2. Interaction of factors on responses. To study interaction effects for independent and dependent variables or factors, 2-dimensional contour plots are analyzed that represent the regression equations. In a contour plot that represents two response factors, one factor is kept constant, and the extent of the interaction determines the shape of the plot. The interaction effect between four factors significantly affecting PHA production was well studied as a 2-D contour plot presented in Fig. 5. In Fig. 5 interactions between AC, AD, BC, BD, and CD show an elliptical contour plot which suggests a positive effect on PHA production with increasing concentration of all factors A, B, C, and D. However, AB interaction shows a negative effect on elliptical contour plot suggesting higher concentration of A and B influences lower PHA production. The experiments were analyzed in a randomized manner. The R^2 value also known as coefficient determines the fitness of the model. The value was 96.56 % at $p < 0.05$ for PHA production, which enables to depiction of

Table 5
Box-Behnken experimental design for optimizing four factors.

Run	Factor 1	Factor 2	Factor 3	Factor 4	PHA concentration (g/L)	
	KH_2PO_4 (%)	Inoculum size (%)	$MgSO_4$ (%)	Temperature (°C)	Predicted values ± 0.024 SD	Experimental values
1	0	+1	−1	0	0.558	0.555 ± 0.012
2	+1	0	0	−1	0.658	0.638 ± 0.013
3	+1	0	+1	0	0.799	0.785 ± 0.026
4	0	0	0	0	0.630	0.624 ± 0.011
5	0	−1	+1	0	0.702	0.692 ± 0.024
6	−1	0	0	+1	0.602	0.599 ± 0.017
7	0	0	0	0	0.630	0.632 ± 0.019
8	0	+1	0	−1	0.548	0.596 ± 0.021
9	0	0	+1	−1	0.620	0.619 ± 0.022
10	0	0	0	0	0.630	0.644 ± 0.026
11	0	0	0	0	0.630	0.669 ± 0.02
12	+1	0	−1	0	0.668	0.671 ± 0.01
13	0	0	0	0	0.630	0.657 ± 0.01
14	−1	0	−1	0	0.462	0.427 ± 0.028
15	−1	0	0	−1	0.452	0.447 ± 0.022
16	+1	−1	0	0	0.740	0.756 ± 0.021
17	0	−1	−1	0	0.571	0.538 ± 0.026
18	−1	−1	0	35	0.534	0.559 ± 0.023
19	0	−1	0	40	0.712	0.735 ± 0.015
20	+1	+1	0	0	0.727	0.752 ± 0.023
21	−1	0	+1	0	0.592	0.579 ± 0.012
22	0	−1	0	−1	0.561	0.549 ± 0.021
23	0	0	−1	+1	0.640	0.667 ± 0.025
24	0	0	0	0	0.630	0.679 ± 0.014
25	0	0	−1	−1	0.489	0.468 ± 0.019
26	0	+1	+1	0	0.689	0.654 ± 0.017
27	0	0	+1	+1	0.771	0.781 ± 0.026
28	−1	+1	0	0	0.520	0.508 ± 0.022
29	0	+1	0	+1	0.699	0.685 ± 0.024
30	+1	0	0	+1	0.800	0.754 ± 0.011

Table 6
Analysis of variance for PHA with four factors.

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	p-value	Significant
Model	0.2566	14	0.0183	30.07	<0.0001	
Linear						
A	0.1275	1	0.1275	209.14	<0.0001	
B	0.0005	1	0.0005	0.8530	0.3703	
C	0.0681	1	0.0681	111.70	<0.0001	
D	0.0512	1	0.0512	84.01	<0.0001	
Square						
A^2	0.0019	1	0.0019	3.11	0.0982	
B^2	0.0001	1	0.0001	0.1478	0.7061	
C^2	0.0005	1	0.0005	0.8611	0.3681	
D^2	0.0032	1	0.0032	5.20	0.0376	
2-way interaction						
AB	0.0006	1	0.0006	0.9058	0.3563	
AC	0.0003	1	0.0003	0.5314	0.4772	
AD	0.0004	1	0.0004	0.5921	0.4536	
BC	0.0024	1	0.0024	3.86	0.0683	
BD	0.0008	1	0.0008	1.24	0.2829	
CD	0.0003	1	0.0003	0.5613	0.4653	
Residual	0.0091	15	0.0006			
Lack of Fit	0.0069	10	0.0007	1.50	0.3416	**Not significant**
Pure Error	0.0023	5	0.0005			
Cor Total	0.2658	29				

A= KH_2PO_4 (%), **B**= inoculum size (%), **C**= temperature (°C), and **D**= $MgSO_4$ (%).
$\mathbf{R^2}$ = 96.56 % and $\mathbf{R^2_{adj}}$=93.35 %.

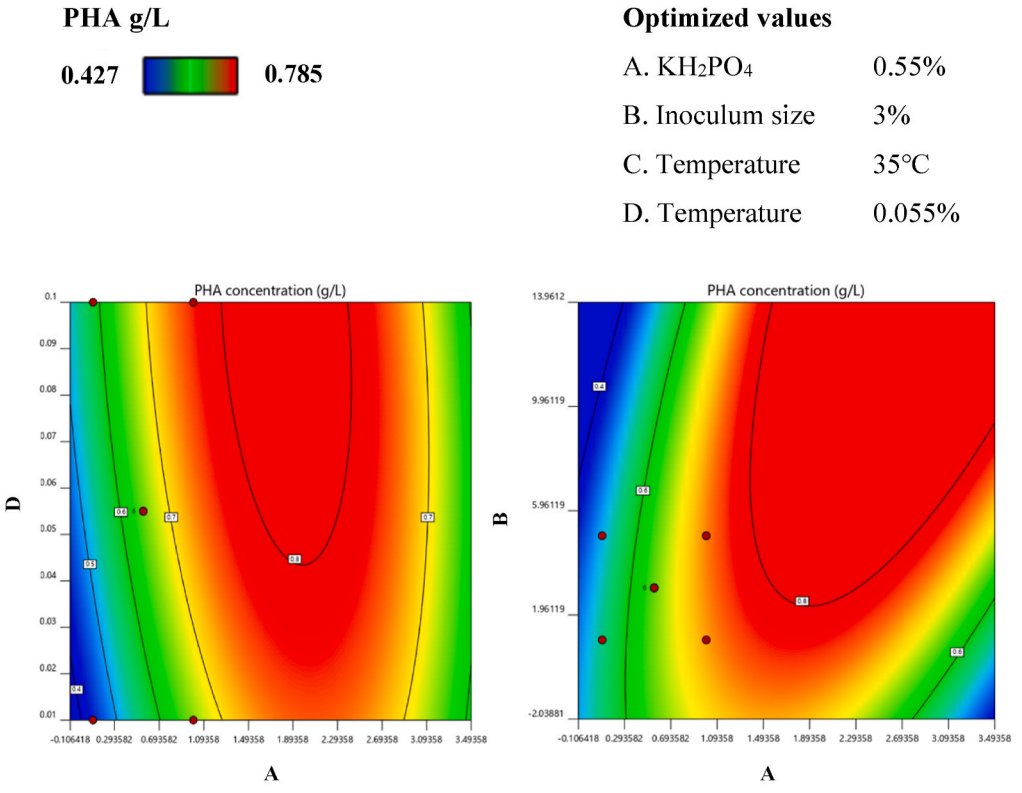

Fig. 5. 2D Contour graphs representing 2-way interaction among A, B, C, and D (KH_2PO_4, inoculum size, temperature, and $MgSO_4$ respectively) on PHA production by *B. paranthracis* RSKS-3. The shape of contour plots signifies how factors influence the response (PHA in this case). Interaction between AD, AC, BC, BD, and CD shows a positively shaped elliptical contour plot which positively enhances the PHA production, whereas, AB interaction shows a negative elliptical contour plot that limits the PHA production.

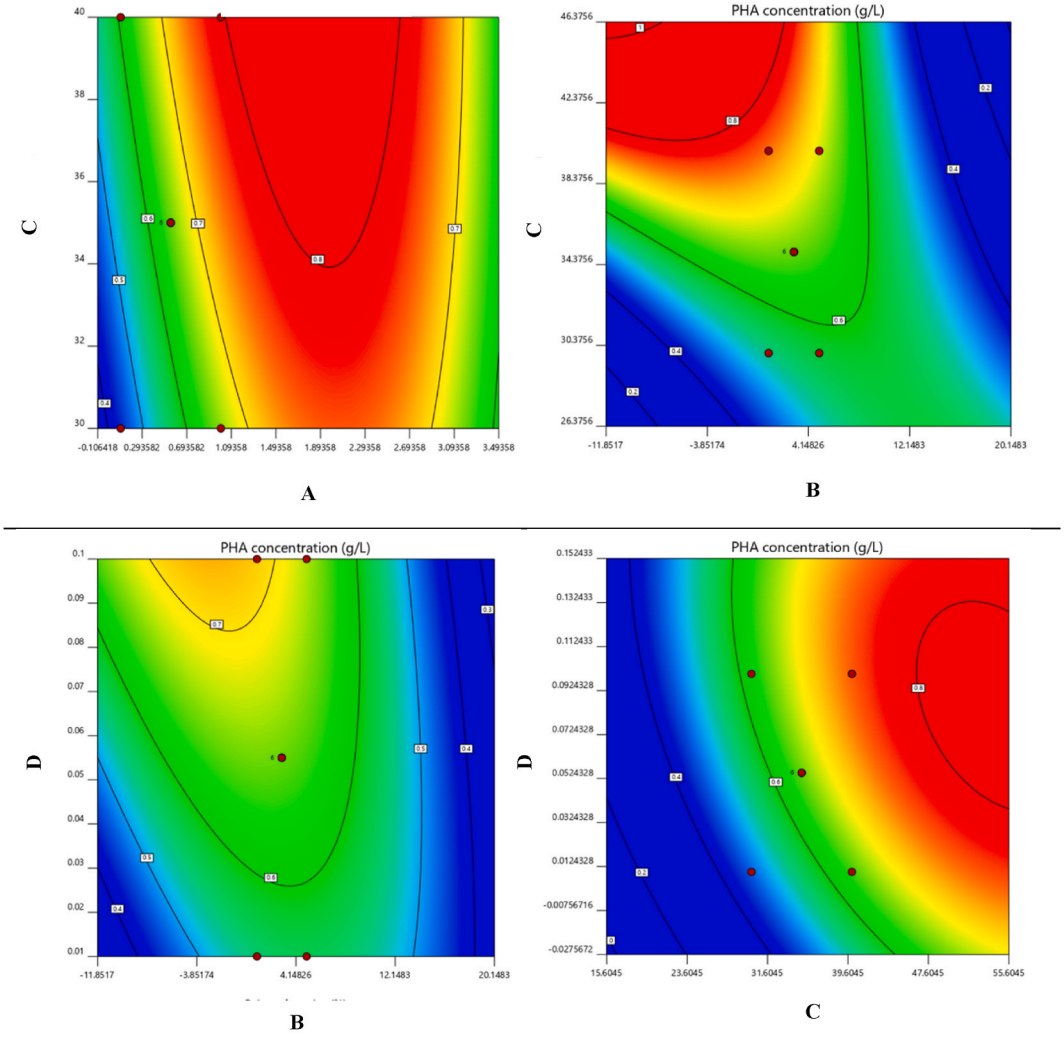

Fig. 5. (*continued*).

response variable identification. Through this model, it was evident to predict optimal PHA production by *B. paranthracis* RSKS-3 which was also analyzed by predicted values provided by the statistical BBD model (Table 6).

So, in our study, an increase in PHA production statistically of about 4.52 g/L was reported under optimized conditions as

Table 7
Comparison of PHA production g/L between unoptimized and optimized conditions. The indication of bold letters corresponds to factors for RSM design.

Factors	Unoptimized conditions	Optimized conditions	PHA content	
			UnOptimized	Optimized
Glucose $(NH_4)_2SO_4$	10 g/L	10 g/L	0.34 g/L	4.52 g/L
KH_2PO_4	3 g/L	3 g/L		
$MgSO_4$	**3.18 g/L**	**5.5 g/L**		
$ZnSO_4$	**0.2 g/L**	**0.55 g/L**		
Na_2HPO_4	0.02 g/L	0.02 g/L		
Peptone	3.8 g/L	3.8 g/L		
Inoculum size pH	1.5 g/L	1.5 g/L		
Temperature	**1 %**	**3 %**		
Incubation period	7.0	7.0		
	37°C	**35°C**		
	72 h	72 h		

compared to the unoptimized condition that yielded 0.34 g/L (shown in Table 7).

A study was conducted by Evangeline and Sridharan (2019), wherein they successfully isolated a strain of *Bacillus cereus*, namely VIT-SSR1, from a site contaminated with industrial effluent. This strain was found to be capable of collecting PHB effectively. The Plackett-Burman design was employed to conduct a screening of the key parameters that influenced the generation of PHA. The study utilized the Response Surface Method to assess the interactive effects among the independent variables. A study was conducted with a Central Composite Design methodology to optimize the quantities of molasses, ammonium sulfate, and starting pH. The study yielded a polyhydroxyalkanoate (PHA) content of (1.42 ± 0.01) g/L and achieved a maximum PHA production of (40.3 ± 0.77)%, utilizing the optimized circumstances [39].

Sabapathy et al. utilized statistical techniques, specifically the Plackett-Burman design (PBD) and Response Surface Methodology (RSM), to assess the key factors that impacted the production yield of PHA. In the preliminary stage, the researchers chose three variables (glycerol, KH_2PO_4, and incubation time) from the Plackett-Burman design to be further optimized using the Box-Behnken design. The researchers conducted a comprehensive analysis of the interplay among these variables, leading to the development of an optimized medium that effectively facilitates the production of large levels of PHA. Before the commencement of the optimization procedure, the concentration of polyhydroxyalkanoate (PHA) was quantified at 0.52 ± 0.05 g/L, accompanied by a cell dry mass (CDM) of 1.07 ± 0.32 g/L. Following the implementation of the optimization, a notable 5.84-fold rise in PHA concentration was observed, resulting in the attainment of 3.04 g/l of PHA [40].

Chmelová et al. (2021) aim to develop a minimum synthetic medium that would maximize biomass yield and optimize specific independent factors through the application of response surface methodology (RSM). The maximum biomass yield, estimated at 1.71 ± 0.04 g/L, was attained in the optimized medium. The optimized medium was composed of glucose at a concentration of 8.4 g/L, sodium ammonium phosphate at a concentration of 5.7 g/L, and a phosphate buffer at a concentration of 35.4 mM. They concluded that under the optimized experimental circumstances, it was observed that the carbon and nitrogen sources were fully depleted after a cultivation period of 48 h. Furthermore, the biomass yield exhibited a significant increase of 1.7-fold in comparison to the conditions employed in earlier experiments [41].

Sehgal et al. conducted optimization research, initially applying the one variable at a time (OVAT) strategy and afterward using the response surface methodology (RSM) approach. The Plackett Burman Design (PBD) was employed to conduct a screening of the major characteristics that exhibited the most prominent influence on the production process. To evaluate the combined and individual impacts of these major variables, the researchers conducted experiments using a central composite design (CCD). Among the many carbon sources derived from agro-industrial waste, rice husk (2 % weight/volume) was determined to be the most favourable for the manufacture of polyhydroxyalkanoates (PHA), resulting in a yield of 1.431 ± 0.06 g/L. The PBD screening process revealed that many factors, including the concentration of nitrogen and carbon sources, the age of the inoculum, and the size of the inoculum, exerted a considerable influence on the formation of PHA. After doing CCD optimization, the researchers achieved a PHA output of 5.13 g/L by utilizing an ideal concentration of rice husk at 3.7 % (w/v), ammonium chloride concentration at 0.15 % (w/v), an inoculum age of 26 h, and an inoculum size of 1.3 % (v/v). This resulted in a significant 2.124-fold increase in production [42].

Hence, challenges in scaling Polyhydroxyalkanoate (PHA) production include optimizing media for strains like Bacillus cereus VIT-SSR1, Pseudomonas oleovorans, and Priestia megaterium POD1, which utilize diverse substrates such as rice mill effluent and agricultural waste. These efforts require overcoming complexities in substrate availability, process efficiency, and downstream applications like biopolymer film fabrication. In this research, Bacillus paranthracis RSKS-3 presents promise with its potential to efficiently

Fig. 6. A PHA film produced by *B. paranthracis* RSKS-3 under optimized synthetic media. A chloroform-dissolved PHA was poured into a watch glass and air-dried using a hot air oven at 60 °C temperatures.

convert glucose into PHA under controlled conditions, suggesting it could offer a solution to scalability challenges through tailored medium optimization and robust metabolic pathways.

4.3. PHA production under statistically optimized conditions

After the screening and optimization of factors through PB and RSM design, production was carried out, and based on the quantity of PHA produced by *B. paranthracis* RSKS-3, Table 7 summarized the comparison between initial unoptimized conditions and optimized conditions after statistical design for PHA production. The increased fold of PHA production after optimization was 13.29-fold.

4.4. PHA identification using UV spectroscopy

The PHA extracted from B. paranthracis RSKS-3 (Fig. 6) was identified using a UV spectrophotometer. Crotonic acid was produced by boiling the PHA pellet in concentrated sulphuric acid, and the resulting absorbance spectrum was analyzed in the range of 800 nm–190 nm. The obtained spectrum was compared with the standard crotonic acid UV spectrum, as depicted in Fig. 7.

4.5. PHA characterization

By using FTIR spectroscopy, functional groups of the isolated PHA granules were analyzed (shown in Fig. 8). FTIR spectroscopy works by measuring the absorption of infrared radiation by a sample, which is related to the vibrational frequencies of the chemical bonds in the sample. By analyzing the pattern of absorption peaks in the FTIR spectrum, it is possible to identify the functional groups present in the sample and to obtain information about the chemical structure and composition.

The FTIR spectrum of PHA typically exhibits absorption peaks corresponding to the carbonyl (C=O) and ester (C–O) functional groups, which are characteristic of the polymer backbone. The peak positions and intensities can provide information about the degree of crystallinity, molecular weight, and composition of the polymer. Specifically, the peaks observed at 1457 cm^{-1}, 779 cm^{-1}, 1751 cm^{-1}, 1000-1300 cm^{-1}, 1155 cm^{-1}, and 2923 cm^{-1} correspond to the stretching of –CH bending, carbonyl (-C=O) esters, various carbon-hydrogen groups (-CH, $–CH_2$, $–CH_3$), carbon-oxygen (-CO) bonds, and –OH stretching, respectively [43–45].

X-ray diffraction analyzed the crystallinity of the materials, PHA in this case, and enables us to understand the arrangement of polymer chains as well as crystalline structures. In our research, the XRD of PHA provides valuable insights into the crystalline structure at specific intensities (Fig. 9). The 2θ values of PHA at the XRD graph exhibit peaks at 27.289°, 29.4°, and 45.354°. A relatively low crystalline index (CI) of 4.59 % at the peak intensity of 27.289 suggests an amorphous phase, a CI of 39.41 % at the peak intensity of 31.639 suggests crystalline structure, and a CI of 24.24 % at the peak intensity of 45.354 also suggests crystalline structure that falls between the extremities of other two values. The values highlight the PHA being a heterogeneous material in nature and influence molecular characteristics and conditional crystalline arrangement. Our findings share equivalent peaks with other studies reported by [46–49].

SEM analysis of polyhydroxyalkanoates (PHAs) offers useful information about the surface morphology and structure of these biodegradable polymers (Antony 2021). This technique enables a thorough analysis of the surface of the material at both micro and nanoscales, uncovering characteristics such as granules, surface roughness, and overall texture. In addition, scanning electron microscopy (SEM) allows for the quantification of particle size and dispersion, providing crucial insights into the uniformity of polyhydroxyalkanoates (PHAs). The acquired images can emphasize distinct surface characteristics, such as pores or fissures, which have

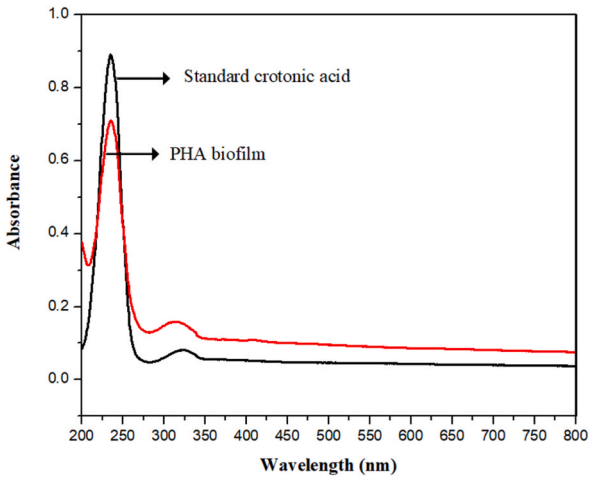

Fig. 7. UV spectra of PHA film produced by B. paranthracis RSKS-3 and standard crotonic acid. The PHA film produced was treated with concentrate H_2SO_4 and converted to crotonic acid. The produced crotonic acid was analyzed as spectra from 800 to 190 nm against standard crotonic acid using a UV spectrophotometer.

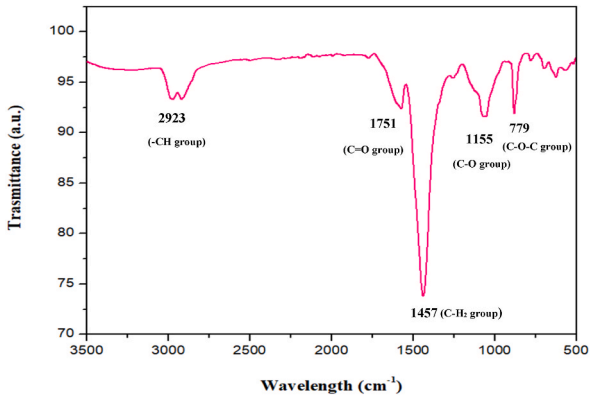

Fig. 8. FTIR of PHA film produced by *B. paranthracis* RSKS-3 under statistically design-optimized conditions. The peaks at 2923, 1751, 1457, 1155, and 779 cm^{-1} correspond to CH, C=O, CH_2, C–O, and C–O–C functional groups.

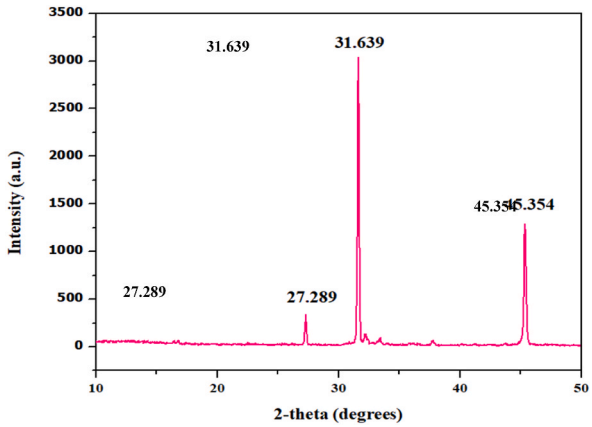

Fig. 9. XRD of PHA film with 3 intensities peaks at 27.289, 31.639, and 45.354°.

an impact on the qualities of the material. The use of SEM is crucial for examining the interaction between PHAs and other substances, particularly in the context of composite materials or blends. Furthermore, it is utilized in degradation studies to analyse alterations in surface morphology over time by comparing images taken before and after degradation. SEM plays a crucial role in quality control during PHA manufacture, guaranteeing uniformity and dependability. The microstructural investigation conducted by SEM helps to

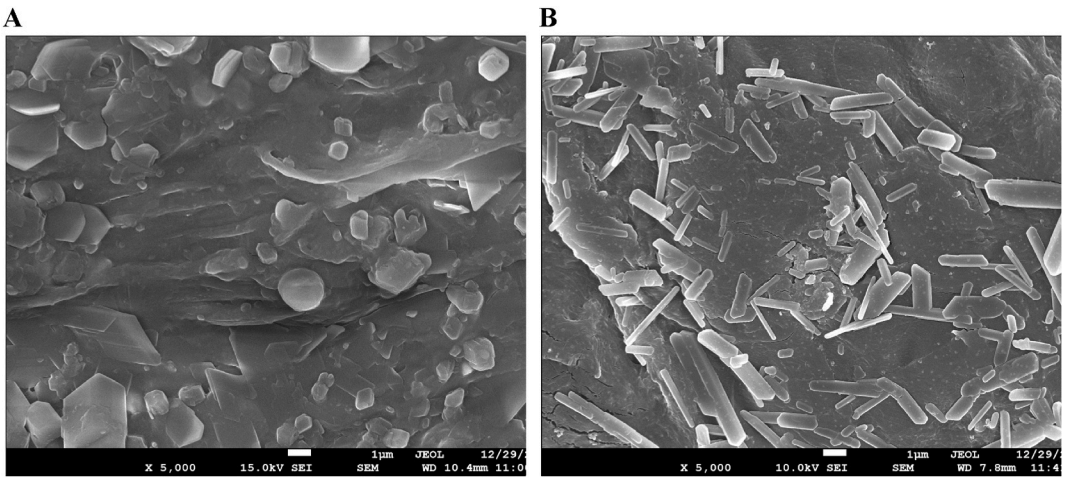

Fig. 10. Scanning Electron Microscopy showing surface morphology of (A) standard PHA and (B) PHA of *B. paranthracis* RSKS-3.

enhance our understanding of PHAs, facilitating their optimization for various applications in sustainable and biodegradable materials.

The structure and shape of standard PHA were analyzed using SEM at a magnification of 2500X (Fig. 10). The observation of Polyhydroxyalkanoates (PHA) under Scanning Electron Microscopy (SEM) reveals distinct microstructures for the extracted and standard forms of PHA. The microstructures of PHA extracted from B. paranthracis RSKS-3 (Fig. 10B) are predominantly rectilinear, indicating an orderly arrangement of the PHA granules produced by the bacterial cells. This rectilinear structure suggests a consistent and potentially crystalline organization of the polymer chains. In contrast, standard PHA (Fig. 10A) displays a variety of shapes ranging from circular to irregular. These varied morphologies likely result from different production and processing methods, with circular shapes indicating spherical granules and irregular shapes representing areas of less uniform polymer chain arrangement [50, 51].

Nuclear Magnetic Resonance (NMR) spectroscopy, which includes proton (H NMR) and carbon-13 (C NMR) studies, is a powerful method for understanding the complex molecular structures of polyhydroxyalkanoates (PHAs) (Kag 2023) (Araneda 2023). H NMR spectroscopy offers comprehensive information about the PHA monomer units, allowing for the identification of specific proton environments and the clarification of the hydrogen arrangement within the polymer chain. The spectrum additionally provides data on the protons located at the ends of the polymer chains, which assists in determining the process of polymerization. In addition, C NMR spectroscopy reveals the many carbon habitats found in PHAs, with peaks corresponding to distinct carbon types that offer crucial information about the composition of the polymer. The chemical shifts seen in C NMR spectra provide additional characterization of carbon atoms present in both the PHA backbone and side chains [52,53].

The monomeric structure of the chemical recovered by *B. paranthracis* RSKS-3 was determined using 1H and 13C NMR spectra, shown in Fig. 11(A and B). The signal at 1.2 ppm in the 1H NMR spectrum was attributed to the solvent, $CDCl_3$. The peaks observed at 3.479 and 3.747 ppm are attributed to the methylene (–CH–(CH_2)–CO–) group adjacent to an asymmetric carbon atom with a single proton. The 1H NMR spectrum displayed signals corresponding to the protons of methane (–CH–) at 5.2 ppm, indicating the presence of -O–(CH–) CH_2- linkage at carbon number 3. Nevertheless, the signal observed between 16.651 and 33.856 in Fig. 11 (B) in the 13C NMR spectrum indicates the existence of methylene groups inside the polymer. The peaks ranging from 63.854 to 77.268 represent the side chain of the PHA polymer. Finally, the signals ranging from 165.730 to 173.313 indicate the presence of carbonyl groups that are connected to ester bonds, which are also present in the polymer.

Thermogravimetric Analysis (TGA) is a significant technique used to understand the thermal properties of polyhydroxyalkanoates (PHAs). TGA entails subjecting the PHA sample to controlled temperature fluctuations while monitoring its weight loss. This research provides significant insights into the thermal stability and decomposition characteristics of PHAs. The initial reduction in weight, commonly associated with the emission of volatile chemicals, indicates the beginning of deterioration (Zhou 2023; bacha 2023). The TGA curve offers a decomposition profile that elucidates the various stages of the degradation process and provides insights into the intricate nature of thermal breakdown. In addition, TGA facilitates the assessment of degradation byproducts, and when used in conjunction with methods such as gas chromatography-mass spectrometry (GC-MS), permits the detection and measurement of distinct breakdown components. Comparative thermogravimetric analysis (TGA) studies assist in evaluating the thermal stability of various polyhydroxyalkanoate (PHA) formulations, hence aiding in the enhancement of polymer characteristics for a wide range of applications. Furthermore, TGA plays a crucial role in quality assurance during PHA manufacturing, guaranteeing uniformity and forecasting the material's behavior under different temperature settings [54].

The thermal stability of PHA granules in the air was determined by thermogravimetry (TGA). Fig. 12 represents the TGA curve of the PHA extracted from *B. paranthracis* RSKS-3 up to 600 °C. The onset of thermal degradation (T_{onset}) was at 388.01 °C, which means that the polymer was stable around 388.01 °C and above this temperature, polymer degradation was initiated. The temperature having maximum sample weight loss (T_{max}) was 436.97 °C, and the maximum weight loss of about 98.01 % was achieved [55].

5. Conclusion

The Plackett-Burman design (PBD) is a highly effective tool for testing the significance of various media components in PHA production. This study employed PBD to screen the media components affecting PHA production by the newly isolated *B. paranthracis* RSKS-3. The experiment results showed that four of the nine variables tested significantly affected PHA production at a 95 % relative significance level. These variables were temperature, inoculum size, KH_2PO_4, and $MgSO_4$. The study revealed that optimizing these four significant variables can enhance PHA production by the novel isolate. Temperature plays a crucial role in microbial growth; hence, it is one of the most important factors to consider during optimization. Inoculum size, on the other hand, can impact the substrate utilization rate and cell growth, affecting PHA production. KH_2PO_4 and $MgSO_4$ are essential nutrients that influence the metabolic activity of bacteria, and their optimization can lead to better PHA production. C-source (glucose) plays a crucial role in the PHA production, however, in this study it was non-significant. The plausible reason may be that the bacteria *B. paranthracis* RSKS-3 optimally utilizes glucose and the factor does not significantly affect the production. Also, our study had a limitation of not including a nitrogen source, as the factor generated noise in the model. Upon optimizing these significant factors through the RSM tool, a maximum PHA production of 4.52 g/L was reported. Overall, the use of PBD and RSM in this study allowed for the rapid screening, identifying, and optimizing multiple media components has enhanced PHA production by the novel isolated *B. paranthracis* RSKS-3.

Ethics approval and consent to participate

This study did not require ethics approval, and no formal consent to participate was obtained as the research did not involve human

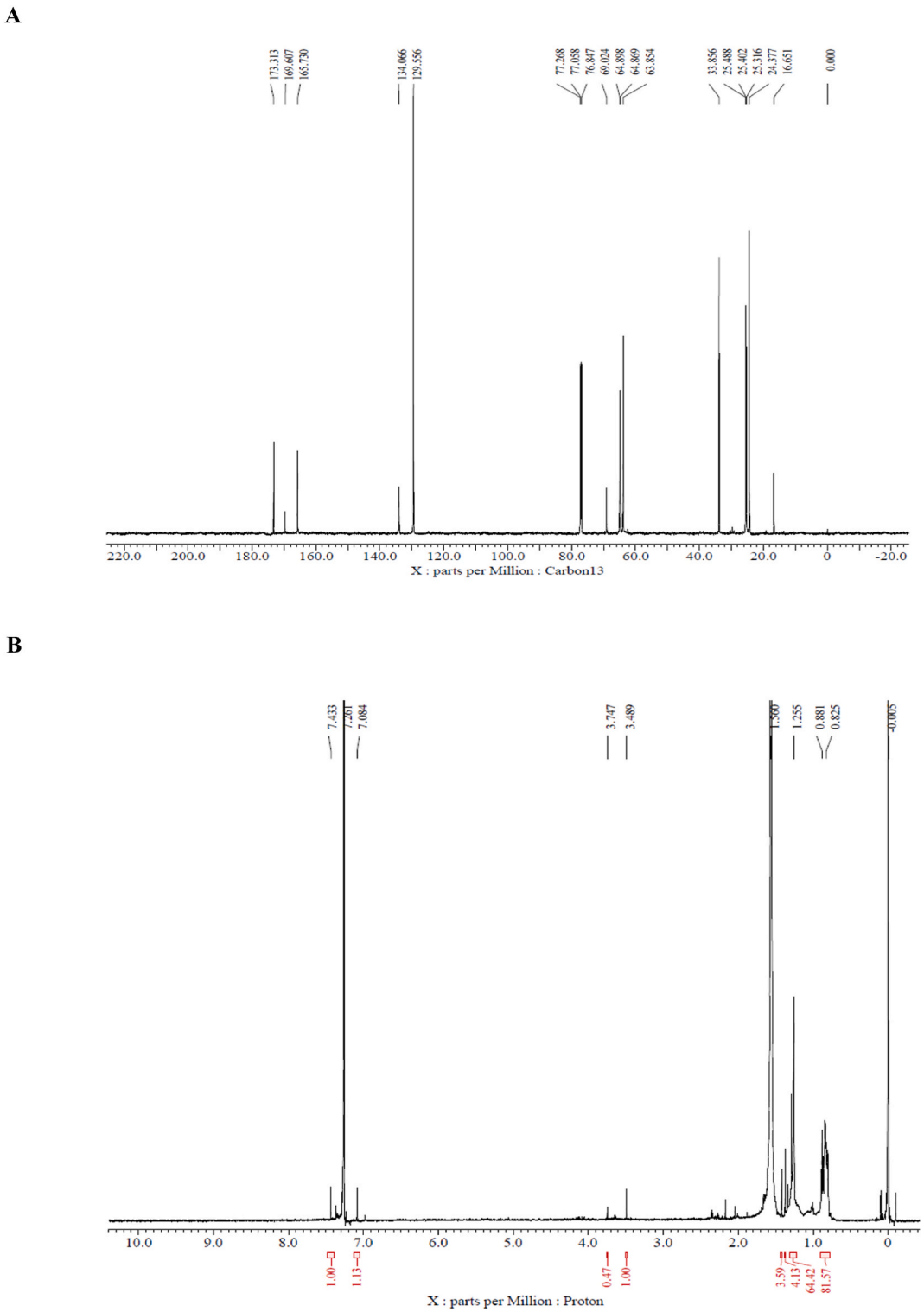

Fig. 11. NMR analysis, where, (A) C′ NMR (B) H′ NMR of extracted PHA from *B. paranthracis* RSKS-3.

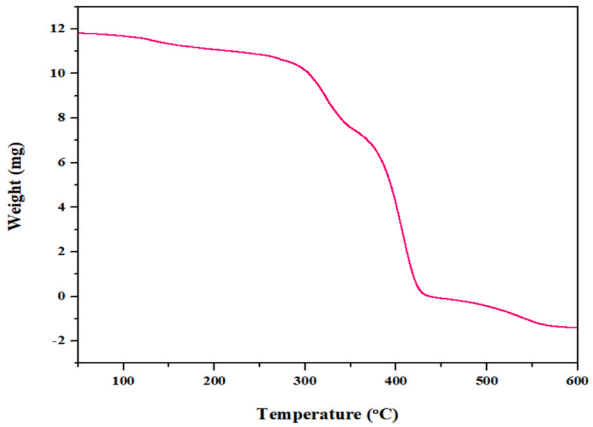

Fig. 12. Thermo-Gravimetric Analysis of extracted PHA from *B. paranthracis* RSKS-3.

subjects or sensitive data.

Consent for publication

Not applicable.

Availability of data and materials

The data and materials supporting the conclusions of this study are available upon request. Interested parties may contact the corresponding author to obtain access.

Competing interest

The authors declare no competing interests that could influence the interpretation of the research findings or the presentation of the results.

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CRediT authorship contribution statement

Rohan Samir Kumar Sachan: Writing – original draft, Methodology, Investigation, Data curation. **Inderpal Devgon:** Writing – original draft, Validation, Resources, Formal analysis. **Abdel Rahman Mohammad Said Al-Tawaha:** Writing – original draft, Visualization, Validation, Resources. **Arun Karnwal:** Writing – review & editing, Writing – original draft, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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