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A Statistical Method for Optimal Production of Polyhydroxybutyrate by *Bacillus flexus* (*Priestia flexa*) SN01023 using Dairy Wastewater as Substrate

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Abstract

Background: The continuous acceleration in plastic consumption has resulted in alarming environmental consequences, petrochemical-based plastics significantly contribute to prolonged ecological degradation. To counter these pernicious effects, polyhydroxybutyrate, being a biocompatible and biodegradable polymer, presents itself as a promising solution. This study introduces Polyhydroxybutyrate production using sustainable and economical feedstock to reinforce the circular economy approach. **Objectives:** In the present study, *Bacillus flexus (Priestia flexa) SN01023* isolated from mangrove soil was used for optimization studies for the highest Polyhydroxybutyrate (PHB). It is attempted to grow this isolate in dairy industrial wastewater and used it as a substrate for PHB production. **Methods:** Response surface methodology (RSM) was used to analyze the interactive effects of pH, temperature, and substrate concentration. The statistical tool BBD was used to optimize these cultural parameters, and the highest PHB production was recorded. The extracted PHB was characterized using GC-MS, TGA, and NMR. **Findings:** The highest PHB production was attained at 7.5 pH, at a temperature of 40 °C with a substrate concentration of 60%, and the maximum PHB accumulation potential of *B. flexus SN01023* can reach up to (4000.210 mg/L). GC-MS peaks displayed monomer chains of biodegradable polyester. TGA curve showed maximum thermal decomposition between 257-288 °C, primarily indicating the thermal stability of PHB. **Novelty:** In this research study, *B.flexus SN01023* isolated from mangrove soil is used for PHB production using dairy wastewater as feedstock, which is an unprecedented approach. And considering the outcomes, it can be concluded that *B. flexus SN01023* can be regarded as a luring agent for PHB production using dairy wastewater more sustainably.

Keywords: *B. flexus*; Dairy wastewater; Optimization; Polyhydroxybutyrate; Response surface methodology (RSM); Box Behnken design (BBD)

1 Introduction

”Plastics” are generally invoked as synthetic or processed polymers primarily obtained from petroleum, and it is non-biodegradable. Plastics have become necessary in several facets of modern life for various applications, including medical, textile, aerospace, construction, electrical equipment, telecommunication, and many other sectors. Nonetheless, their reasonable cost often expands broad applicability and leads them to be discarded uselessly. According to assessed data, between 4.8 to 12.7 million tonnes of plastic material in landfills end up in oceans every year, and due to the lack of proper waste management strategies, it is projected to increase by 1.3 billion tonnes by 2040[(1)]. This governed the pursuit of bio-based plastics as an alternative, which is environmentally safe, sustainable, and biodegradable. Microbial bioplastics (PLA and PHAs), plants (cellulose-based and starch-based), and a variety of renewable raw feedstocks (polysaccharides) can all be used to make bioplastics.

Among them, Polyhydroxyalkanoates (PHAs) are invariably outstanding bioplastics due to non-toxicity, biodegradability, and biocompatibility aspects and also have comparable properties to petroleum-based plastics. PHAs are polyesters accumulated intracellularly by numerous microbes as energy storage material produced through bacterial fermentation with comparable physicochemical characteristics to conventional plastics. The most prevailing PHA is Polyhydroxybutyrate (PHB), which many microorganisms synthesize by binding β-hydroxybutyrate monomers with ester bonds, which is exceptionally marketed owing to its ecologically safe properties, non-toxicity, thermoplasticity, crystallinity and compostability[(2)]. Regardless of potential advantages, there is not enough development in PHB commercialization due to its high production cost. Raw materials cover the maximum part of the overall production cost[(3)]; this directly impacts the generation and utilization of PHB as the whole process questions the economic feasibility. Its colossal production cost has to be reduced to build PHB as an absolute substitute for petroleum-based plastics, using numerous wastewater streams as organic feedstocks/substrates.

This purpose could be accomplished by designing sustainable and feasible biopolymer generation methods using the ”waste to resources approach.” PHB can be produced from complex organic feedstocks incorporating waste stream ones, such as agro-industrial wastewater[(4)], wood mill effluent[(5)], pulp and paper industrial wastewater[(6)], milk whey and dairy wastewater[(7)], industrial products such as crude glycerol[(8)] to sewage sludge. Therefore, this will have a substantial role in plastic's circular economy, as we can recover resources from wastewater treatment operations. The main research gap in study of efficient PHB production is that several other potential substrates for PHB production are yet to be discovered, therefore this carried study proposes a novel venture encompassing reuse of dairy effluent into worthful biopolymer, this research study is first attempt to use *B. flexus SN01023* as strain and dairy wastewater as feedstock/Substrate for PHB production and optimization of different parameters using BBD to attain maximum results.

The primary advantage of using RSM is that it compresses the trial numbers needed to calculate variable interactions[(9)], and this ensures a systematic understanding of experimental outputs, conversely pertaining to PHB production. This study is a demonstration of PHB production using dairy industrial wastewater as an economical nutrient source. PHB generation on an extended scale using this approach is a valuable and useful way of disposing of dairy wastewater. Dairy industrial wastewater is strenuously enriched with carbohydrates, proteins, and fats from milk. Due to the high organic load of dairy effluent, it can be an efficient substrate for bacterial growth and PHB production. Using this wastewater would alleviate the binary challenge of wastewater management and plastic accumulation. Accordingly, in our study, we attempted to use dairy wastewater as a feedstock/Substrate for PHB production and optimized different parameters to attain maximum results. The experimental results of this study signify that *B. flexus SN01023* is a potential producer of PHB, utilizing industrial dairy wastewater as feedstock.

2 Methodology

2.1 Procuration of culture

The bacterial strain used for the PHB production in the present work was procured from a mangrove soil sample (21°40′ N, 72°17′ E) and phylogenetically identified by 16S rRNA gene sequencing as *Bacillus flexus SN01023 (Priestia flexa)*. The strain was preserved at 4 °C on agar slants and sub-cultured regularly to ensure viability.

2.2 Pre-treatment of Dairy Wastewater

The milk facility of Vita in Rohtak (Haryana), India, provided the dairy effluent, which was stored at 4°C until further examination. To remove the undesired suspended material, the effluent was filtered using a muslin cloth and rough filter paper. Only after this pre-treatment step, the effluent was used as a substrate for the selected PHB-producing isolate.

2.3 PHB extraction and quantification

PHB extraction was carried out as per the method of Alarfaj et al. (2015) [10] with slight changes. The substrate with accumulated biomass was centrifuged for 8-10 minutes at 3000 rpm. Acetone and ethanol were added to the procured pellet of cells in a 1:1 ratio in order to rupture the cell wall and liberate PHB. The pellet was then spun for five minutes at 8000 rpm, and the supernatant was disposed of. Following a 30-minute period at ambient temperature and an 8-minute spin at 8000 rpm, the resulting pellet was liquified with sodium hypochlorite. The polymeric granules were then liquified with chloroform and sieved through Whatman filter paper and weighed accordingly.

2.4 Statistical Optimization of Growth Parameters for maximal PHB-production

The optimization of various parameters to achieve maximal PHB production using the selected isolate was conducted by employing dairy effluent as the substrate. The chosen optimum value for each parameter was maintained constant for subsequent experiments. The influence of several culture conditions, including substrate concentration (20-100%), pH (4-11), and temperature (30-50°C), was assessed to determine accumulated biomass and PHB production (Table 1). The parameters were improved utilizing the standard Response Surface Methodology (RSM) approach with Box-Behnken design (BBD) to enhance PHB generation. A BBD was conducted utilizing Design Expert software version 8.0.4.1 (State Ease, Inc., Minneapolis, USA) to clarify the interplay of these cultural aspects on PHB development. A total of 17 experiments were conducted across three levels: low (-1), medium (0), and high (+1). The design matrix derived from the Box-Behnken Design (BBD) included minimum, medium, and maximum levels of selected variables for the experimental sets. In this context, PHB production (weight) served as the dependent variable (response), while pH, temperature, and substrate concentration were the independent variables. The three-dimensional contour graphs were employed to assess the interplay of cultural characteristics and their impact on responses. RSM is an empirical statistical technique employed to analyze the association between controlled parameters and observed responses, used to improve media elements and other factors necessary for the synthesis of biomolecules. Optimization of various parameters for the highest PHB production was performed using software (Design Expert, ver. 8). The statistical tool BBD, one type of RSM which employs use of three levels per input factor and lays the output to a quadratic model, was applied to optimize cultural parameters such as Substrate concentration, pH and temperature and to enhance PHB accumulation in *B. flexus*. These critical variables were prescribed into three levels (coded -1, 0, +1), and a design matrix of 17 experimental runs was obtained, represented in (Table 2). The Attained experimental responses and predicted values were calculated by applying ANOVA to critically check if the polynomial expression is competent to anticipate recorded responses statistically or not (Table 3). The following second-order polynomial equation was obtained by optimizing these three critical elements with three centre points in BBD:

PHB Content = +3993.40-122.25* A-207.00 * B+205.25* C+198.75* A * B-36.75* A * C+195.25 * B * C-1490.58* A2-319.58 * B2-816.57* C2

Three-dimensional response surface plots of PHB production were made for each variable's optimum level (Figure 1). PHB accumulation was significantly influenced by substrate (< 0.0001) either independently or in relation to one another. The acquired p-values and lack of fit values (<0.0001) further favour that the obtained experimental responses truly fit the model; the predicted and attained experimental responses for PHB production were firmly comparable. The 3D surface plots distinctly showed continuously declining PHB production with linearly rising temperature and pH. It was observed in 3D plot that PHB production progressively increased up to 60% concentration and decreased further. Predicted values for PHB production were computed by applying regression analysis and linked with experimental response data, which evidently represents that obtained experimental response values and predicted response values are in total agreement. The ANOVA was tabulated (Table 3) for the obtained model, which confers that variables pH temperature and substrate had the significant effect ($p<0.001$); the value of total determination coefficient ($R = 0.9998$) shows that the model fits the obtained experimental data coherently. Additionally, the model's validity as exceptionally significant is confirmed by the adjusted determination coefficient value (Adj. $R2 = 0.9995$).

Table 1. Factors with chosen range and acquired levels

Factor with range	Low-Level	Mid-Level	High-Level
pH (4-11)	4	7.5	11
Temperature (30-50 ^{o}C)	30	40	50
Substrate Conc. (%)	20	60	100

2.5 Structural Analysis

2.5.1. Gas Chromatography-Mass spectrometry (GC-MS)

GC-MS is a potent technique for understanding and investigating the structural information of a compound. Analysis was performed using the methodology given by Lee and Choi, 1997 with slight modifications[11]. For methanolysis of extracted PHB, 5mg of it was dissolved in an already prepared mixture of 1ml methanol, 2 ml chloroform and 1ml of 3% H2SO4, and the solution was kept at 100 °C for 2hrs in an ampoule vial to carry out the hydrolysis, finally after cooling 1ml of distilled water was added into acquired solution, Subsequently organic phase containing methyl esters of monomer was collected for analysis. The sample was analyzed using a Gas chromatography Mass Spectrophotometer (GC-MS QP-2010S M Shimadzu Japan), nitrogen was used as carrier gas (1ml/min), and the programmed temperature used was 45 °C for 7 min, with a temperature ramp of 4°C/min up to 100°C.

2.5.2. Thermogravimetric analysis (TGA)

The TGA HiRes1000 instrument was used to perform thermal degradation of the extracted PHB. Prior to analysis, the sample was vacuum-dried for 24 hours at 35°C. Measurements were made at temperatures ranging from 34°C to 600°C at a rate of 20°C per minute. A mass% vs temperature graph was created, and the thermal deterioration pattern was simultaneously analyzed.

2.5.3. Nuclear Magnetic Resonance (NMR)

According to Narayanan et al. (2021) NMR spectroscopy is a powerful and non-destructive tool for examining the synthesis and breakdown of polymers; its main advantages are speed, accuracy, and sensitivity[12]. NMR examination of extracted PHB was performed at the sophisticated instruments facility, Punjab University, Chandigarh. 1HNMR spectra were recorded by dissolving 5mg of extracted PHB in deuterated chloroform (CDCl3), and analysis was carried out on a BRUKER AVANCE NEO 500 MHz NMR SPECTROMETER. The spectrum was determined at 25 °C with 3 seconds of pulse repetitions, and the acquired spectrum was compared with already-reported literature.

3 Result and Discussion

3.1 Effect of pH and temperature

The highest PHB accumulation in *B. flexus SN01023* was attained at a temperature of 40 °C and pH of 7.5, 3D surfaces and contour plots for PHB accumulation with differing pH and varying temperature were developed. Accumulation of PHB was constantly elevated with a simultaneous rise in temperature of the substrate and reached the highest value at 40 °C. Based on temperature and substrate, the contours and 3D response surface plots were made while keeping the temperature at (0 level), and it was established that PHB accumulation could significantly increase with a continuously escalating substrate concentration. It was evidently observed that PHB accumulation was significantly elevated with a linear increase of pH up to 7.5, which implies that a further rise in pH would not affect PHB accumulation. PHB production is often growth dependent, so temperature optimization aids maximum biomass growth for efficient usage of substrate[13], as temperature affects metabolism, cell membrane fluidity, and nutrient assimilation. In the temperature range between 37-40 °C, multiple bacteria attain a balance between accelerated growth and metabolic stress evasion. In a study performed by Ali et al. using *Bacillus licheniformis* IIB-isl9 and its mutant NA-cys4, it was observed that temperatures beyond 45 °C can cause thermal stress that hampers PHB production. Similarly, Trakunjae et al. (2021)[14] also optimized temperature using *Rhodococcus pyridinivorans* BSRT1-1 and demonstrated that high temperature often disturbs cell growth and enzyme activation, which challenges PHB accumulation. The pH of the medium scrupulously affects the catalytic activity of enzymes, which collectively ascertains the capability of a bacterium to produce PHB. At nearly neutral pH (≈7), primarily all bacterial cells maintain intracellular proton balance and achieve a homeostatic state. Enzymes responsible for PHB production, such as acetoacetyl-CoA reductase and PHA synthase, work efficiently at a balanced pH for efficient biopolymer generation. Mostafa et al. (2020) investigated that pH 7.5 yields maximum PHB production using marine isolate *Pseudodonghicola xiamenensis* and illustrated that this slightly basic pH was optimum for enzymatic and metabolic activity[15]. Similarly, Balasubramanian et al. (2024) using *Enterobacter hormaechei* reported that the highest PHB production occurred at pH 7.5 under experimental conditions[16].

3.2 Effect of substrate concentration

B. flexus SN01023 was grown on different concentrations of dairy wastewater, such as 20%, 40%, 60%, 80% and 100%. Among the different substrate concentrations, 60% was found to be the most appropriate concentration for effective PHB production.

B. flexus SN01023 grew luxuriantly at a concentration of 60%, while lower growth occurred at other concentrations. These results are in concordance with a study conducted by Policastro et al. (2020) using winery wastewater, it was found that PHB production was susceptible to initial substrate concentrations[17]. Very low substrate concentrations restricted carbon flux, while exceedingly high concentrations provoked metabolic instability. Similarly, Hendy et al. (2025) examined PHB production using municipal wastewater sludge, and it was suggested that beyond a threshold, increased substrate concentrations do not correspondingly increase PHB accumulation due to oxygen imbalance and metabolic saturation[18]. Tourang et al. (2023) also investigated PHB production using agro-industrial waste, and the study outcomes demonstrated that substrate load has a compelling impact on PHB yield, as low concentrations do not provide enough carbon flux to the cells and high concentrations induce inhibitory effects on accumulation[19]. The relationship between pH and temperature, in which decreasing temperature and increasing pH, PHB production accomplished a quadratic gain (Figure 1a). Likewise, (Figure 1b) displays the interaction between substrate and pH by keeping the temperature at zero level. Up to the definitive point, the PHB content increased steadily with continuous increase in both of the specified variables above zero however, close to the boundary there was a keen convergence of curve, which explains that PHB production in substrate would not be affected by pH above certain limit (Figure 1b). Furthermore, the relationship between substrate concentration and temperature was found to be precisely significant. Out of all three parameters, PHB production was significantly affected by substrate concentration, followed by pH and temperature. From the above-mentioned individual parameter study, it can be established that optimum conditions for PHB production using *B. flexus SN01023* were as follows: pH: 7.5, Temperature: 40 °C, and Substrate concentration: 60%.

Table 2. Box Behnken experimental design matrix with coded values

Run	pH	Temperature (°C)	Substrate (%)	Predicted Value (mg/L)	Actual Value (mg/L)
1	0.000	0.000	0.000	3993	4000
2	-1.000	1.000	0.000	1899	1921
3	1.000	0.000	-1.000	1395	1398
4	0.000	0.000	0.000	3993	4000
5	0.000	1.000	-1.000	2249	2235
6	0.000	1.000	1.000	3050	3032
7	1.000	0.000	1.000	1732	1739
8	-1.000	-1.000	0.000	2711	2699
9	0.000	0.000	0.000	3993	3967
10	0.000	-1.000	-1.000	3054	3073
11	1.000	1.000	0.000	2052	2065
12	-1.000	0.000	-1.000	1556	1560
13	0.000	-1.000	1.000	3074	3089
14	0.000	0.000	0.000	3993	3967
15	0.000	0.000	0.000	3993	4000
16	1.000	-1.000	0.000	2069	2048
17	-1.000	0.000	1.000	2050	2048

3.3 Model Competency Investigation

It is imperative to evaluate the well-fitted model to ensure it accurately reflects the existing actual system. The precision of the fitted model depends on the coefficient of determination (R^2). A higher R^2 value indicates superior prediction of experimental responses based on data outcomes. The R^2 value for the acquired model was determined to be 0.99, indicating that 99% of the variation in the response can be adequately described, while suggesting that 1% of the fluctuations occurred during the experiments and were not accounted for by the model. The model was validated through trials conducted in triplicate under optimum conditions.

3.3.1. GC-MS

The GC-MS chromatogram of the extracted PHB exhibited a significant peak with a retention time of 18.56 minutes, indicating the presence of the isopropyl ester of 2-butenoic acid, thus confirming the existence of PHB. The area obtained from the peak indicates the PHB concentration, measured at 41.8%. Comparable findings were documented in the research conducted by

Table 3. ANOVA for Response Surface Quadratic Model

Source	Sum of squares	Df	Mean square	F-value	p-value
Model	1.470E+007	9	1.634E+006	3454.89	< 0.0001*
A-pH	1.196E+005	1	1.196E+005	252.87	< 0.0001*
B-Temperature	3.428E+005	1	3.428E+005	725.00	< 0.0001*
C- Substrate	3.370E+005	1	3.370E+005	712.80	< 0.0001
AB	1.580E+005	1	1.580E+005	334.18	< 0.0001
AC	5402.25	1	5402.25	11.43	0.0118
BC	1.525E+005	1	1.525E+005	322.52	< 0.0001
A^2	9.355E+006	1	9.355E+006	19785.79	< 0.0001
B2	4.300E+005	1	4.300E+005	909.48	< 0.0001
C2	2.808E+006	1	2.808E+006	5937.97	< 0.0001
Residual	3309.70	7	472.81		
Lack of Fit	2438.50	3	812.83	3.73	0.1179 not significant
Pure Error	871.20	4	217.80		
Cor Total	1.470E+007	16			
Std. Dev.	21.74				
Mean	2757.29				
C.V. %	0.79				
PRESS	40377.25				
R-Squared	0.9998				
Adj R-Squared	0.9995				
Pred R-Squared	0.9973				
Adeq Precision	155.776				

El-Kadi et al. (2021), which involved the characterization of PHB from various bacterial strains[20]. The Peaks at retention times 7.8 and 11.8 represent dodecane, Tetradecane, and decanoic acid; these compounds show that monomer chains of this extracted PHB are of the biodegradable polymer family. GC-MS chromatogram of PHB is shown in Figure 2; these results are in concordance with a study carried out by Khang et al. (2021)[21].

3.3.2. TGA

TGA gives knowledge relating to chemical phenomena such as desolvation, decomposition, chemisorption, and solid-gas reactions. Thermogravimetric analysis (TGA) for extracted polyhydroxybutyrate (PHB) was conducted within the temperature range of 34-600°C. Maximal mass breakdown of 92.82% of PHB was recorded at 288°C, with initiation occurring about 257°C, likely due to β-elimination, which is responsible for ester cleavage processes in the PHB molecule[22]. TGA curve of extracted PHB is shown in Figure **??**, Hassan et al. (2019) recorded rapid thermal degradation between 260 and 290°C, taking an extracted PHB sample produced by *Bacillus subtilis* using agricultural and industrial wastes, which is almost similar to the attained results in this study[23]. Considering the thermal stability of PHB, this biopolymer can possibly be utilized in extensive-scale production of bioplastics, and the current study indicates that strategically using dairy wastewater can provide a thermally resistant biopolymer in an economically lucrative way.

3.3.3. NMR

NMR spectroscopy is a primary approach to effectively examining the structure of extracted PHB. The ^{1}H NMR spectra of PHB showed chemical shift signals at 1.29 and 1.28 ppm, which correspond to the methyl group of hydroxubutyrate's molecule. Similarly, a set of quadruplets at 2.46-2.64 ppm, which is typically a distinguishable character of the methylene group (-CH2) attached to the carbonyl group, identical results were reported by Feng et al. (2023) while characterizing synthesized PHB from the actinomycetes *Aquabacterium sp*. A7-Y[24]. In the spectra, multiple signals were recorded at 5.29-5.25 ppm, a particular feature of the methine group (-CH). Similar findings were reported by Mostafa et al. (2020), while characterizing PHB extracted from *Erythrobacter aquimaris*[25], and with ^{1}H NMR result, the presence of polyhydroxybutyrate was concluded. The acquired peak at 7 ppm is of residual chloroform used in the NMR analysis, NMR spectra is shown in Figure 4. Similar results were

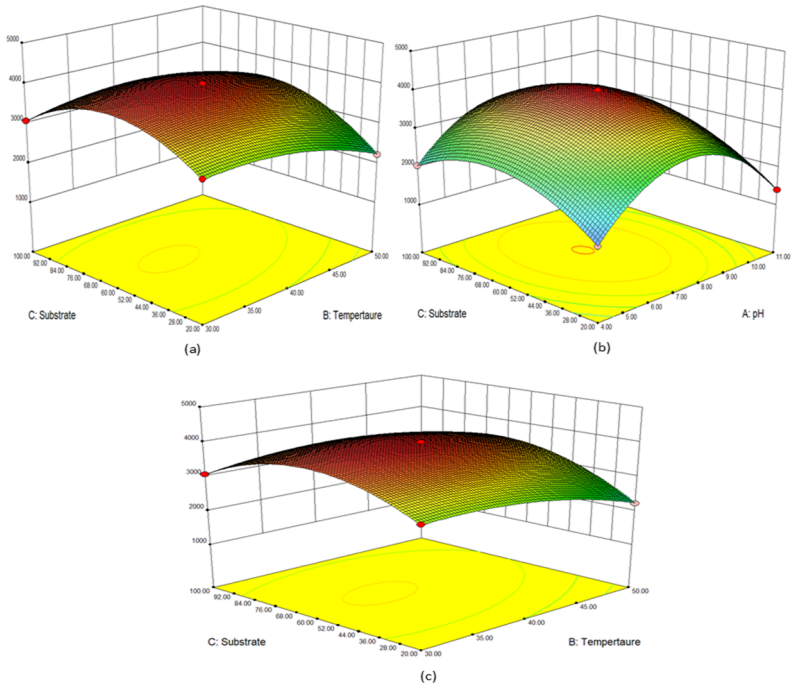

Fig 1. Response surface plots displaying the effect of cultural parameters on PHB production

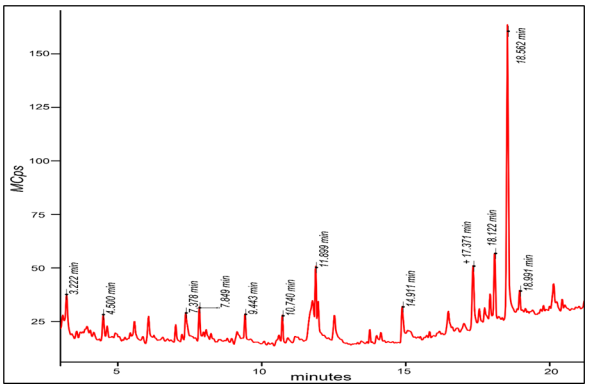

Fig 2. GC-MS spectra of extracted PHB from *B. flexus* SN01023

noted by Battashi et al. (2019) during ^{1}HNMR analysis of synthesized PHB by *Burkholderia sacchari* using wastepaper as substrate [26]. Identical results were noted by Mostafa et al. (2020) in the structural assessment of PHB accumulated by bacterium *Pseudodonghicol axiamenensis* [27].

4 Conclusion

The profuse use of petroleum-based plastics causes harmful effects on the environment and human health due to their biodegradation-resistant potential. Hence, there is an urgent need for biopolymers that are environmentally safe to counteract plastics. The current study concentrated on the production of biopolymer (PHB) utilizing a cheap carbon source for the growth of PHB-producing bacteria. In this study, *B. flexus SN01023* was selected to optimize cultural parameters to attain the highest PHB content using inexpensive dairy wastewater as feedstock. The PHB accumulating *B. flexus SN01023 strain* was procured from mangrove soil. Mangroves are a potent source of the accumulating microbial community due to high salinity and variation in nutrient availability. Marine environments like the mangroves support a variety of microorganisms that produce biopolymers; these microorganisms have a distinct set of genes and enzymes. Reported results displayed the significant potential of *B. flexus*

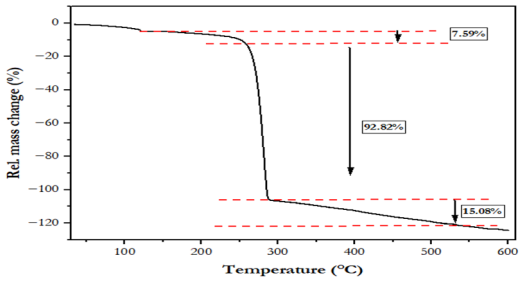

Fig 3. TGA graph of extracted PHB from *B.flexus* SN01023

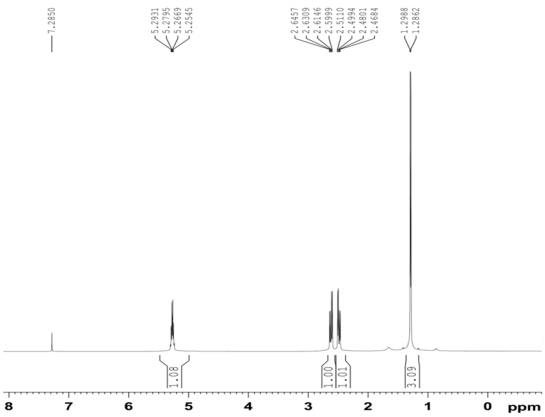

Fig 4. ^{1}H NMR analysis of extracted PHB from *B. flexus* SN01023

SN01023 to produce PHB up to 4000.210 mg/L. Extracted PHB samples were characterized using GC-MS and TGA, which characteristically represent that extracted PHB is a biodegradable polymer and can withstand temperatures up to 288 °C. The concluding outcome of the conducted study is that *B. flexus SN01023* has the efficiency to produce PHB using enormously cheap dairy wastewater as a carbon source. Presently, this strain is subsequently studied to enhance PHB production by studying the outcomes of the scale-up process, and carbon-nitrogen supplementation in dairy wastewater is further under progress. This kind of strategy will improve our ability to unveil industrially unexploited microbes and use them in resource recovery. The attained yield using wastewater, integrated with biopolymer's structural analysis, delivers a robust foundation for waste valorization. While dairy industrial wastewater was successfully effective for PHB production in the current work, investigating other co-substrate or a blend of it with other waste material, such as agro-industrial effluents, food processing waste, and glycerol, etc., may amplify the efficiency of PHB production. The mentioned recommendation lights the way towards scaling the process and the integration of sustainability in economic bioplastic production.

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