Utilization of chicken feathers for PHA accumulation by bacteria isolated from slaughterhouse soil.

Shareen Fatima Rizvi[1], Aamina Shahab[2], Gaurav Yadav[3], Roohi Roohi[4*]

[1,3]Department of Biosciences, Integral University, Lucknow, Uttar Pradesh, India.
[3],[4*] Department of Bioengineering, Integral University, Lucknow, Uttar Pradesh, India.

***Corresponding Author:** Dr. Roohi,
*Department of Bioengineering, Integral University, Lucknow, Uttar Pradesh, India.
Email: roohi0607@gmail.com

Abstract
The goal of this research is to reduce the high manufacturing costs of PHA and make this polymer truly commercially competitive with products derived from fossil fuels. In the present work, soil samples from bird farms and slaughterhouses were collected to isolate bacterial strains with the potential of utilising chicken feathers as a substrate in powder and hydrolysate form along with glucose for accumulating PHA when grown under nutritively limited media. Based on the colour intensity of stain taken up by the bacterial isolates on the application of Nile red and Sudan black B when grown on agar plates with nutrition-deprived media, 2 out of 30 isolated strains (LS18 and LS33) were qualitatively screened to be potent producers of PHA. LS18 was studied biochemically and physiologically followed by its 16S rRNA sequencing. Pre-treatment of chicken feathers for preparing hydrolysate and powder was done before its usage in the production media as a substrate for quantitative screening for PHA production. LS18 and LS33 were found to be capable of utilizing chicken feathers in the form of powder as well as hydrolysate.

Keywords: Plastic pollution, polyhydroxyalkanoates, chicken feathers, valorisation

Statement and Declaration

1. Introduction

The creation and manufacturing of biodegradable polymers has attracted a lot of attention in response to issues with plastic waste accumulation. Polyesters known as polyhydroxyalkanoates (PHA) build up as inclusions in a range of bacteria (Chen et al., 2015). PHA have material qualities that are comparable to those of synthetic polymers now in use and are fully biodegradable upon disposal, making them attractive candidates for biodegradable plastics due to their intrinsic biodegradability. Certain bacterial genera collect polyhydroxyalkanoates (PHAs) as reserve material in case the culture media becomes unbalanced due to an excess of carbon source and low oxygen, nitrogen, phosphorus, sulphur, or magnesium. (Mascarenhas et al. 2017). In imbalanced growth conditions, bacteria can be induced to accumulate PHA, i.e., when the feed substrate's C: N ratio is higher, when oxygen content is low, or when nutrients like nitrogen, phosphorus, or sulphate become limited. PHB is the most well-characterized form of PHA and is accumulated by a wide range of microorganisms. It has been discovered that several bacteria, including Azotobacter, Bacillus, Archaebacteria, Methylobacteria, and Pseudomonas, produce PHA to varied degrees (Pei et al., 2022). Because *Ralstonia eutropha* (previously known as *Alcaligenes eitrophus*) can accumulate PHAs up to 80% dry weight, it has been the focus of a great deal of published studies. Unlike many "so-called" biodegradable polymers created synthetically, PHA-derived plastics have been reported to be biodegradable in both aerobic and anaerobic environments. Although other forms are possible, poly-betahydroxybutyric acid (PHB) and poly-beta hydroxyvaleric acid (PHV) make up the majority of PHAs (Roohi et al., 2024). In the stationary phase of growth, bacteria capable of synthesizing PHA accumulate PHA, and these PHA granules aid in the survival of cells during unfavourable conditions (Inoue et al. 2021).

2. Materials and Methods

2.1 Media Used

SMedia (Screening Media): (Nutrient agar, Glucose 1%)
PEMedia (PHA Enrichment Media): (Urea 1 g/l, Yeast extract 0.16 g/l, KH_2PO_4 1.5 g/l Na_2HPO_4 4 g/l, Mg $SO_4.7H_2O$ 0.52 g/l, $CaCl_2$ g/l).

2.2 Sample collection and Isolation

Soil samples were aseptically collected from a slaughterhouse in Lucknow city, 1g of soil sample was dispensed in 10ml of sterile distilled water to get a dilution of 10^{-2} to 10^{-9}. For the detecting PHA-positive colonies, 0.1ml of each dilution was plated onto an SMedia with Nile Red dye (0.5 µg/ml) which acted as a screening dye for the PHB producers. Plates were incubated at 30°C for 2-3 days, and colonies appeared were observed under UV (Sakai et al. 2015).

2.3 Screening by Sudan Black B straining

The viable colony method of screening with Sudan Black B dye qualitatively assessed all bacterial isolates for PHA production. The nutrient agar medium was enhanced with 1% glucose were streaked with selected isolates. The colonies were coated with a solution of Sudan Black B, which consisted of 0.02% ethanol. The plates were then left undisturbed for a duration of half an hour. Subsequently, the plates were cleansed using ethanol to eliminate any surplus dye present in the colonies. The colonies exhibiting a dark blue colouration were considered to be indicative of positive PHA production. (Phanse et al. 2011).

2.4 Identification of Bacterial Strains

The molecular characterization i.e., PCR amplification and sequencing, using 16S rRNA sequence amplification performed at Bio-kart India Pvt. Ltd, Bangalore. A forward primer: 5′GGATGAGCCCGCGGCCTA3′ and reverse primer: 5′CGGTGTGTACAAGGCCCGG3′ were used to amplify the gene. The PCR temperature cycling conditions were as follows: initial denaturation at 94 °C for 3 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and elongation at 72 °C for 2 min. The final cycle was followed by extension at 72 °C for 7 min. The amplification products were purified under the following conditions: Initial Denaturation: 96°C for 5 min Denaturation: 96°C for 30-sec Hybridization: 50 °C for 30-sec Elongation: 60 °C for 1.30 min. The GeneBank database in the BLAST program of the National Centre for Biotechnology Information was used to compare the sequence of the 16S rRNA gene, which was deposited in GenBank. The phylogenetic tree was constructed using the Weighbor software. LS18 and LS33 were subjected to biochemical studies. The biochemical tests used for identification were the Indole production test, Methyl red test, Voges-Proskauer test, Catalase test, Starch hydrolysis, Casein hydrolysis test, Motility test and Carbohydrate fermentation (Li et al. 2016).

2.5 Pre-treatment of chicken feathers and preparation of hydrolysate and powder

Chicken feathers were collected from the local butcher shop. These were soaked in detergent water overnight and then washed, this process was repeated until all the blood and dirt were removed. The feathers were sun-dried and crushed into coarse powder for further use. For the preparation of hydrolysate, 25g of CFP was boiled overnight in double distilled water. The content was filtered and filtrate was used for the study.

2.6 PHA production using chicken feathers, chicken feather hydrolysate and glucose

Loopful of 24-hour-old bacterial culture was inoculated in 10ml of PHA enrichment media (PEMedia) and incubated for a further 24hr at 32ºC. After 24hrs this was transferred to the media (Urea 1 g/l, Yeast extract 0.16 g/l, KH_2PO_4 1.5 g/l Na_2HPO_4 4 g/l, Mg $SO_4.7H_2O$ 0.52 g/l, $CaCl_2$ g/l) was supplemented with 25g of chicken feather powder/ 4g of glucose/ 15ml of CFH. The media was then incorporated with a trace element solution consisting of ZnSO5 0.13 g/l, $FeSO_4.7H_2O$ 0.02 g/l, and H_3BO_3 0.06 g/l solution. The PHA manufacturing experiment was carried out using 500 ml Erlenmeyer flasks filled with 250 ml of production media. The inoculated flasks were incubated in a rotatory shaker with 60 rpm at 32ºC for 72 hrs.

2.7 Extraction of PHA

The production medium was centrifuged in a refrigerated centrifuge at 10,000 RPM for 15 min at 15°C, followed by thorough washing of it using distilled water. The pellet is then lyophilized for 48 hours and weighed as Dry Cell Weight (g/L) (Aramvash, 2016). With some modification from (Pati et al. 2020), the lyophilized pellet of PHB was treated with 10 mL NaOCl (4%) and incubated at room temperature for 2 hrs. The cell extract obtained was centrifuged at 10,000 rpm for 15 min and then washed sequentially with distilled water, acetone, and ethanol (96%). After washing, the pellet was dissolved in 10 mL chloroform and incubated overnight at room temperature.

3. Results

3.1 Dye-based screening of PHA-producing bacteria from soil

The screening results of PHA accumulation, determined by the Nile-red assay, revealed that 15 bacterial colonies displayed robust fluorescence signals under the gel documentation system (Model BIO-RAD, Gel Doc XR+, U.S.A in contrast to the rest of the colonies (Fig. 1). These were picked and stained using Sudan black b. Out of all the isolates, 6 tested positive in Sudan staining and 5 isolates in Nile red staining. Three strains were found to give positive results for Nile-red as well as Sudan Black stain Table 1. Two of them (LS18 and LS33) were selected based on stain intensity, for further study (Fig. 2). Fluorescence is contingent upon the age of the cell and the quantity of PHA stored within the cell. LS18 took deeper and more prominent than LS33 (Fig. 3).

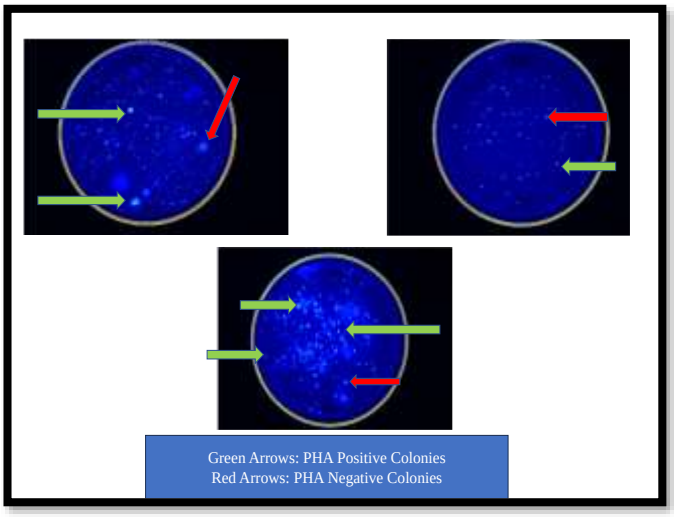

Figure 1. ` Bacterial colonies from slaughterhouse soil on agar plates supplemented with Nile Red under Gel Doc.

Table 1. Nile Red(NR) and Sudan black(SB) staining test of bacterial colonies for screening of PHA producers

Isolates	Nile Red	Sudan Black
LS1	+	-
LS2	-	+
LS7	-	+
LS11	-	-
LS15	+	-
LS18	+	+
LS20	+	-
LS22	-	+
LS23	+	-
LS25	+	-
LS28	+	-
LS30	-	-
LS31	-	-
LS33	+	+
LS34	+	+

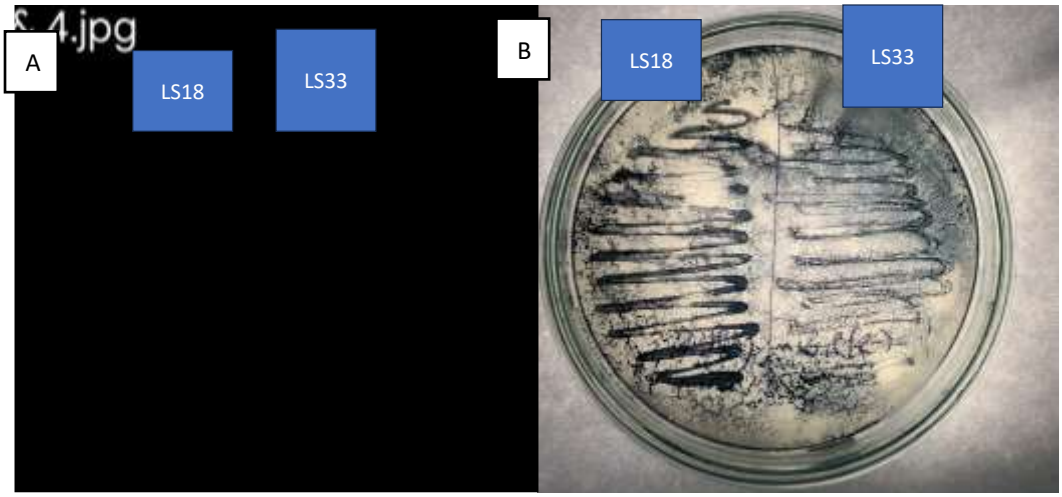

Figure 2. **(**A)Sudan black and (B)Nile Red staining of LS33 and LS18

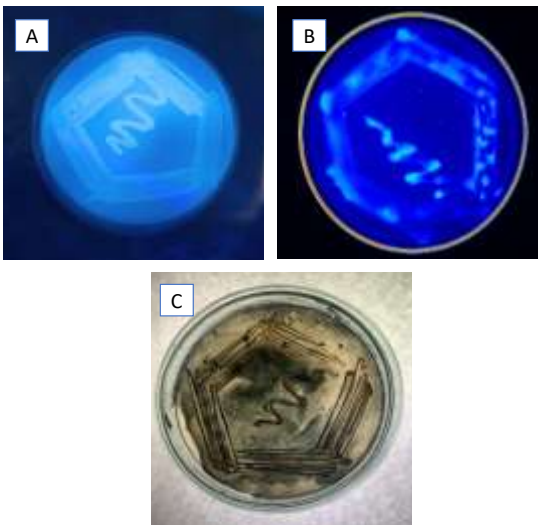

Figure 3. Images of LS18 for PHA selective staining (a) UV chamber (b) Gel Doc. (c) Sudan Staining

3.2 Characterization of LS18 and LS33

Based on the analysis conducted using the BLAST program in the GenBank database, it has been determined that the two isolates can be classified as Actinobacteria. Further, the phylogenetic analysis confirmed that the isolates belonged to the Microbacterium genera LS18 and LS33 as *Rhodococcus qingshengii* with 100% similarity and *Leucobacter komagatae* with 98% similarity respectively (Fig. 3). LS18 and LS33, have had their partial 16S rRNA gene sequences submitted to the NCBI GenBank under the accession numbers PP082742 and PP079216, respectively. The phylogenetic tree of the isolates LS18 and LS33 are depicted in Fig.4 and Fig.5. Table 3. Describes results the biochemical and morphological analysis of LS33 and LS18.

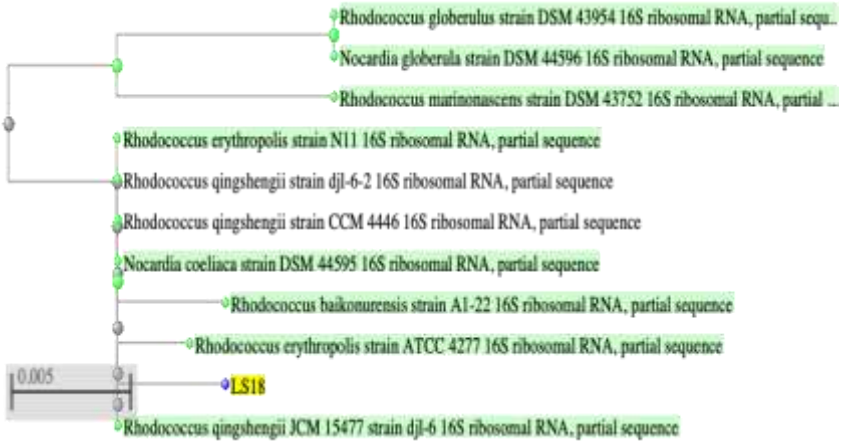

Figure 4. Phylogenetic tree of LS18

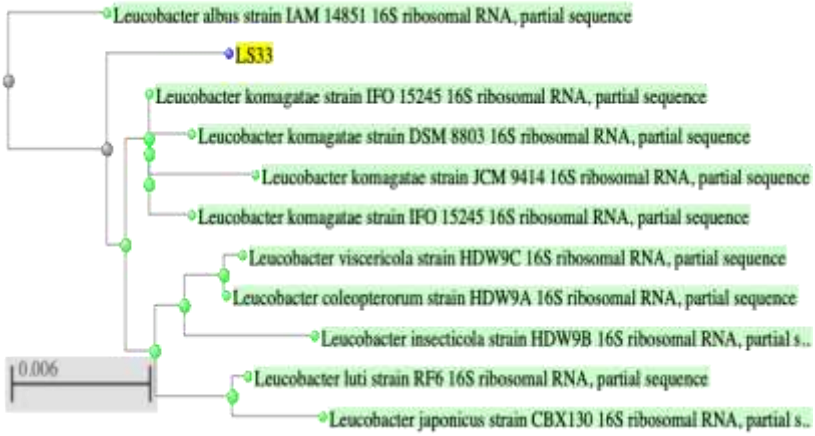

Figure 5. Phylogenetic tree of LS33

Table 2. Biochemical characterisation and Morphological characterisation

Biochemical Tests	**LS18**	**LS33**
Amylase	-	-
Indole	-	-
Motility	-	-
Catalase	+	+
Methyl red	+	-
Casein Hydrolysis	-	-
VP	+	+
Urease	+	+
Glucose Fermentation	+	-
Morphological Tests		
Growth	+++	+++
Elevation	Raised	Raised
Surface	Smooth	Rough
Margin	Slightly Curved	Even
Pigmentation	Pale Yellow	Yellow

3.3 Extraction of PHA Using Chicken Feathers

The PHA produced after fermentation by *Rhodococcus* (LS18) and *Leucobacter* LS33 was 1.02 and 0.81g/L on SNL with 2% glucose respectively, and 2.09 and 1.24 g/L on SNL with 20% chicken feather hydrolysate (Table 3). Remarkably, all samples exhibited a greater synthesis of PHA when exposed to a 10% concentration of chicken feather hydrolysate compared to 2% glucose and chicken feather powder. Nevertheless, the outcome showed great potential for biotechnological purposes when employing chicken feathers hydrolysate for producing PHA. Fig.4 is the stepwise illustration of the production of PHA starting from the preparation of CFH to the extraction of the polymer.

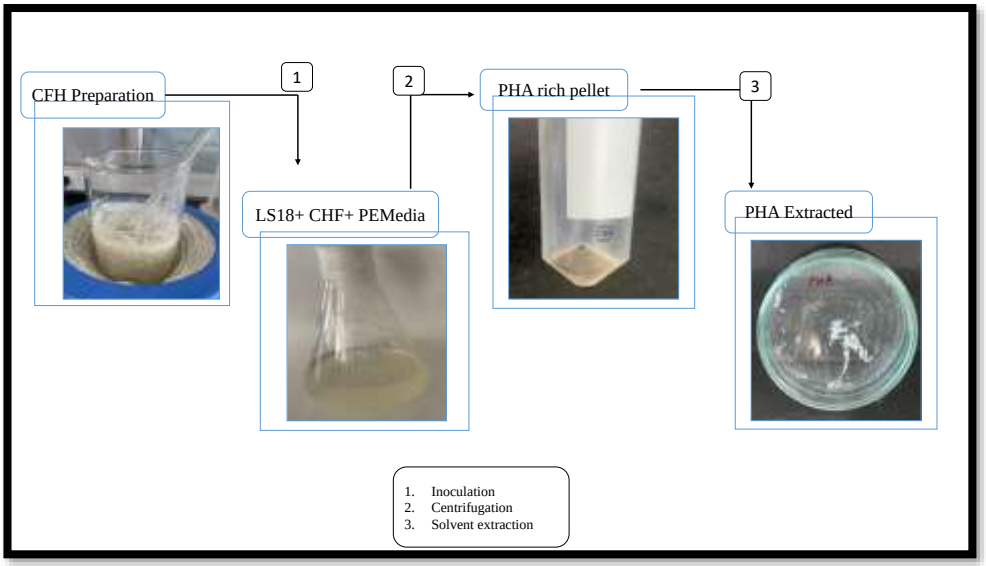

Figure 4. A stepwise process followed for the extraction of PHA from LS18 using CHF. (1) Inoculation of LS18 in PEMedia supplemented with CFH. (2) Centrifugation of PHA-rich media after incubation. (3) Extraction of polymer using solvent extraction method.

Table 3. PHA (g/L) from LS18 and LS33 with glucose, CFH and CFP

Isolates	**PHA g/L**		
	Glucose	**CFH**	**CFP**
LS18	1.02	2.09	1.73
LS33	0.81	1.24	1.61

Discussion & Conclusion

The research on biologically generated polymers has significantly intensified due to the growing concern about the detrimental impact of petrochemical-based plastics on the environment. Due to the competition between synthetic plastics and biopolymers for cost-effective production, the current research work emphasizes the concept of wealth from waste and projects a simple and adaptable way for chicken feather waste valorisation. In this study, our focus was on addressing

the high manufacturing costs of polyhydroxyalkanoates (PHA), aiming to enhance its commercial competitiveness against fossil fuel-derived polymers. In the present study, strains isolated from slaughterhouse soil LS18 and LS33 are showing efficient PHA production. Biochemical and physiological analyses, alongside 16S rRNA sequencing, revealed LS18 and LS33 belonged to the Microbacterium genera as *Rhodococcus qingshengii* and *Leucobacter komagatae* respectively. Several studies have reported Actinobacteria as a producer of PHA polymers (Shah et al. 2007; Narayan et al. 2022; Feng et al. 2023). Goh et al., 2012 also used Nile Red and Sudan Black for staining PHA-producing bacteria. The pre-treatment of chicken feathers, crucial for hydrolysate and powder preparation, significantly contributed to the success of PHA yield. LS18 demonstrated substantial PHA production in flask culture systems, employing a chloroform extraction procedure. The utilization of chicken feathers as a substrate not only showcases an environmentally sustainable approach but also holds promise for cost reduction in PHA production. Our findings contribute to the broader goal of making PHA commercially competitive, and future studies could explore optimization strategies and scalability to further enhance the economic viability of this environmentally friendly polymer. Ultimately, these isolates have the potential to be suitable contenders for the industrial manufacturing of PHA using waste materials. In the future, we plan to improve the media optimization process for enhancing the yield of PHA and utilizing the extracted polymer in diverse biotechnological applications. This optimization will involve both manual and statistical approaches.

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