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ISOLATION OF POLYHYDROXYBUTYRATE PRODUCING BACTERIA FOR ECO-FRIENDLY BIOPLASTIC PRODUCTION

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Abstract

The growing plastic pollution crisis highlights the urgent need for sustainable alternatives such as polyhydroxybutyrate (PHB), a biodegradable polymer. In this study, PHB-producing bacteria were isolated from organic wastes, dumpsite soil, and dairy products. Out of 55 bacterial isolates, five isolates were confirmed as PHB producers.16S rRNA gene sequencing identified them as *Bacillus subtilis*, *Chryseobacterium* sp., and *Stenotrophomonas* spp. PHB was extracted using sodium hypochlorite digestion and characterized by FTIR spectroscopy. Among the isolates, *B. subtilis* KS-24 and *Chryseobacterium* sp. KS-25 produced the highest PHB yields (45 mg/ml [41.7% DCW] and 39.75 mg/ml [38.8% DCW], respectively). Optimal production was achieved at 37°C, pH 7, with glucose as the carbon source and ammonium sulfate or peptone as nitrogen sources after 72 hrs incubation. To the best of our knowledge, this is the first report of *Chryseobacterium* sp. as a PHB producer. These findings highlight promising microbial candidates for industrial-scale bioplastic production, offering an eco-friendly solution to plastic pollution.

Introduction

Plastics are synthetic polymers which have diverse range of uses from domestic, packaging, aquatic and architectural industries due to their lightweight and durability (Andrady and Neal 2009). However, their non-biodegradable nature leads to environmental accumulation and global pollution (Pilapitiya and Ratnayake 2024). To mitigate the environmental challenges associated with conventional plastic waste, attention has shifted toward developing biodegradable plastics produced from renewable feedstocks, collectively referred to as bio-based polymer (Haq *et al.* 2024). Among them, polyhydroxyalkanoates (PHAs) are a class of biopolymers synthesized by bacteria during fermentation of sugar and lipid (Grousseau *et al.* 2013).

Polyhydroxybutyrate (PHB), a member of the PHA family, has attracted significant interest due to its biodegradability, biocompatibility, and thermoplastic properties (Alia *et al.* 2016). Under nutrient-limited but carbon-rich conditions, microorganisms accumulate PHB as an intracellular energy reserve (Bharti and Swetha 2016). PHB biosynthesis has been documented in over twenty bacterial species, including *Methylobacterium rhodesianum*, *Bacillus megaterium*, *Alcaligenes eutrophus*, *Sphaerotilus natans*, and *Escherichia coli* (Heo *et al.* 2008). PHB is readily degraded by various microorganisms via PHB depolymerizes, yielding environmentally harmless products (Choi *et al.* 2004). In addition, the high flexibility and low crystallinity of PHB offer it highly adaptable for processing, thereby indicating its potential for the applications in industrial fields such as materials for packaging (Khanna and Srivastava 2005). Moreover, in the medical field, recent advancements have emphasized on the biosynthesis and advancement of antimicrobial substances incorporating silver nanoparticles and PHB nanocomposites (Castro-Mayorga *et al.* 2018). Despite these benefits, PHB production remains costly compared to petrochemical plastics (McAdam *et al.* 2020). Identifying efficient PHB-producing bacteria and optimizing growth conditions are essential to improving productivity and reducing costs.

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Considering all these facts, the current investigation focuses on isolating novel PHB-accumulating bacteria from various sources, including organic waste, dumpsite soil, and dairy products in and around Dhaka City. The study also aims to characterize the extracted PHB and optimize culture conditions to enhance PHB yield.

Materials and Method

A total of 8 samples of organic waste, soil, and dairy products were collected from various sites within the Dhaka Metropolitan Area, including the Arboriculture Center, dumpsite areas at Jagannath Hall and Shivbari (University of Dhaka), Kolabagan, Ananda Bazar, the drain site near Fazlul Huq Muslim Hall, DU, the Botanical Garden (Jahangirnagar University), and Katabon. After collection, the samples were labeled accurately and brought into the laboratory as soon as possible. Sampling was conducted over a three-month period from July to September 2023. Experimental work, including microbial isolation and characterization, was carried out in the Cell and Tissue Culture Laboratory at the Centre for Advanced Research in Sciences (CARS), University of Dhaka. One gram of each sample was diluted with sterile distilled water and spread onto Luria-Bertani Agar (LBA) and nutrient agar (NA) plates, followed by incubation at 37°C for 72 hrs. Preliminary screening for PHB was performed using an alcoholic solution (0.3%, w/v) of Sudan Black B (SIGMA-ALDRICH, Lot#MKBBQ9075V), following Ansari and Fatma (2016). After staining for 30 min, excess dye was removed, and plates were washed 95% ethanol. Colonies appearing dark blue were considered PHB producers. Sudan Black B staining following Sharma and Dhingra (2015) was used for confirmatory analysis.

Genomic DNA from the PHB-positive isolates was extracted following Sambrook and Russell (2001). Partial amplification of the 16S rRNA gene was carried out using the primers 27 F and 1492R (De Lillo *et al*. 2006). PCR conditions included initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 94°C for 1 min; annealing at 55°C for 30 sec, and extension at 72°C for 1 min; followed by the final extension at 72°C for 5 min. Purified sequences were analyzed using NCBI BLAST, and phylogenetic trees were conducted using MEGA 11.

PHB extraction followed Arshad *et al.* (2017), PHB-positive isolates were grown in Minimal Davis Medium at 37°C for 72 hrs. Ten milliliters of each culture were centrifuged at 6000-rpm for 10 min, and the pellet was treated with 4% NaOCl and hot chloroform at 37°C for 1 hr. After centrifugation at 3000 rpm for 10 min, and the bottom chloroform was collected, and PHB was precipitated using equal volumes of ethanol and acetone. Extracts were dried at room temperature and weighed. Residual biomass was calculated by subtracting PHB dry weight from dry cell weight (DCW). PHB accumulation (%) was calculated as (PHB/DCW) × 100.

For FTIR analysis, 1 mg of the extracted PHB was dissolved chloroform. After solvent evaporation, the polymer film was analyzed using a Shimadzu IRPrestige-21 spectrophotometer between 4000-700 cm^{-1}. Optimization of PHB production was evaluated using different carbon sources (1%): glucose, sucrose, lactose, and starch; nitrogen sources (0.5%): peptone, yeast extract, ammonium sulfate, and ammonium chloride; pH levels (5, 6, 7, and 8); temperatures (30, 37 and 45°C); and incubation periods (24, 48, 72, and 96 hrs). Cultures were grown in 50 ml Minimal Davis broth in 250 ml flask. PHB content was determined following Arshad *et al.* (2017).

Results and Discussion

A total of 55 bacterial isolates were obtained, of which 5 isolates (KS-24, 25, 29, 33, and 45) tested positive for PHB. Sudan Black B staining produced bluish-black colonies (Fig. 1A), and intracellular granules confirm PHB production (Fig. 1B). Sequence analysis identified KS-24 as *Bacillus subtilis*, KS-25 as *Chryseobacterium* sp., and KS-29, KS-33, and KS-45 as

Stenotrophomonas spp. A neighbor-joining phylogenetic tree was constructed using MEGA 11 confirmed their taxonomic placement (Fig. 2). While PHB production by *B. subtilis* is well documented (Hassan *et al.* 2019, Joseph and Chithira 2021), this appears to be the first report of PHB production by *Chryseobacterium* sp.

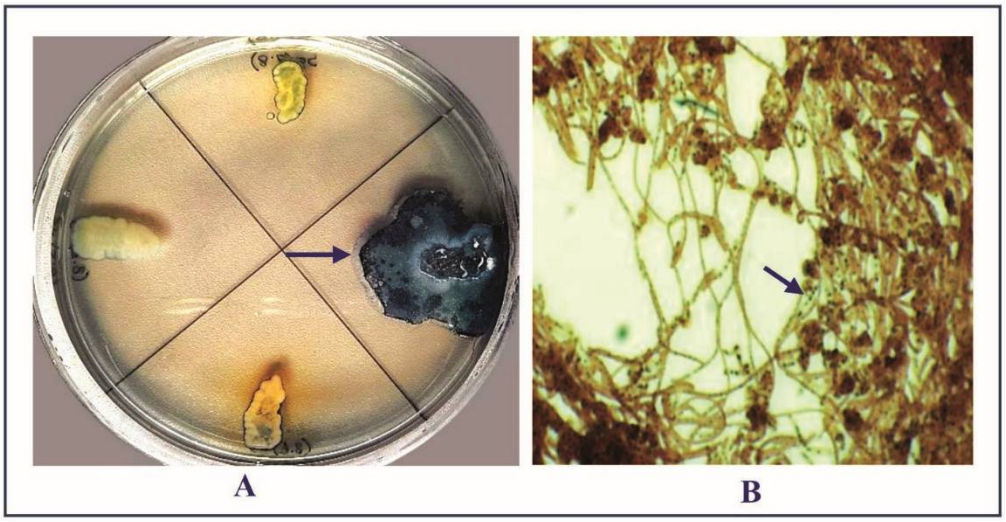

Fig. 1. Screening of PHB-producing bacteria using Sudan Black B. A: Bluish-black colonies indicating PHB production, B: Intracellular PHB granules visible under microscopy.

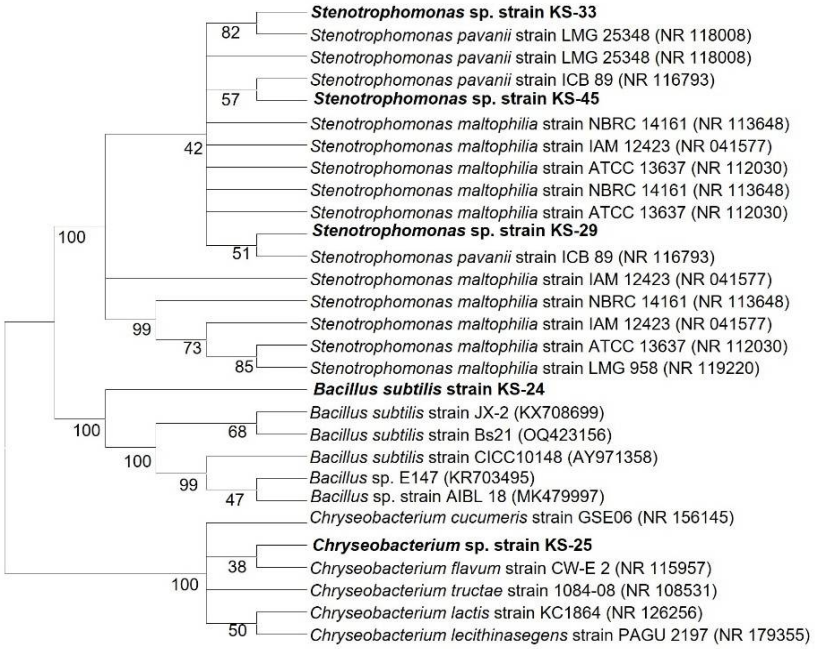

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships of five PHB-producing isolates with closely related species.

PHB extraction and quantification revealed variable yields across isolates. *B. subtilis* KS-24 exhibited the highest PHB production (45 mg/ml), followed by the *Chryseobacterium* sp. KS-25 (39.75 mg/ml) (Table 1). FTIR spectra of isolates KS-24 and KS-25 showed characteristics peaks corresponding to PHB functional groups (Fig. 3). The strong ester carbonyl peak at ≈1740 cm^{-1} confirmed the PHB presence (Alia *et al.* 2016), along with C-O and C-C stretching were found between 1285-970 cm^{-1} (Bayarı and Severcan 2005).

Table 1. PHB production profiles of selected isolates.

Bacterial isolates	Cell dry weight (mg/ml)	Dry weight of PHB (mg/ml)	Residual biomass (mg/ml)	PHB Accumulation (%)
Bacillus subtilis KS-24	108.00	45.00	63.00	41.7
Chryseobacterium sp. KS-25	102.50	39.75	62.75	38.8
Stenotrophomonas sp. KS-29	78.05	24.60	53.45	31.5
Stenotrophomonas sp. KS-33	74.37	21.25	53.12	28.6
Stenotrophomonas sp. KS-45	84.00	21.00	63.00	25.0

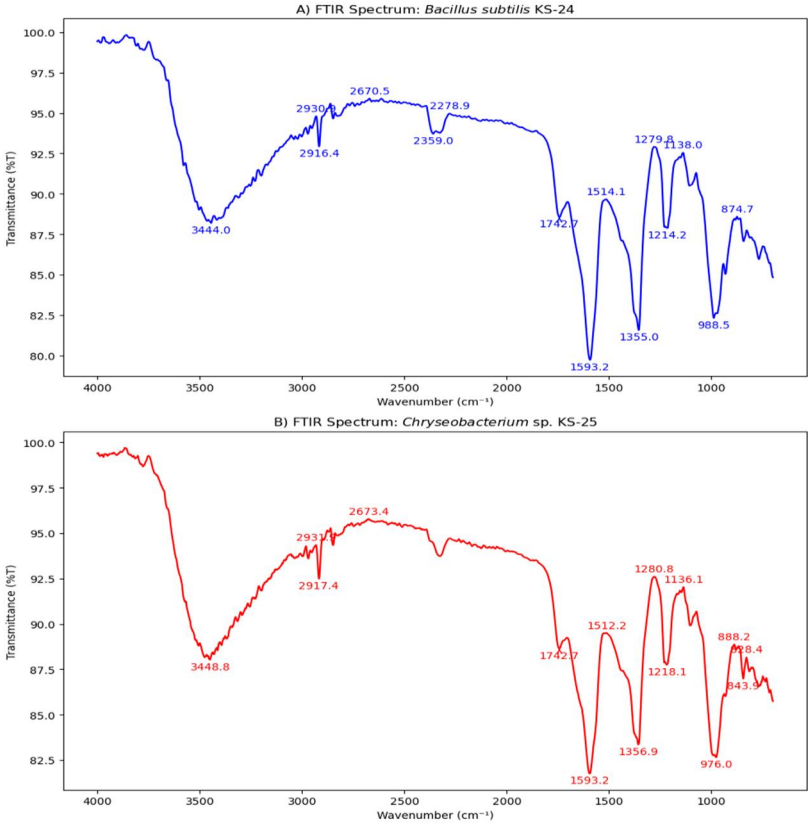

Fig. 3. FTIR spectra confirming PHB structure. A: *Bacillus subtilis* KS-24, B: *Chryseobacterium* sp. KS-25.

Optimization studies showed glucose as the most effective carbon source, yielding 60.3 mg/ml PHB in KS-24 and 41.75 mg/ml in *Chryseobacterium* sp. KS-25 (Fig. 4A), consistent with earlier studies (Hori *et al.* 2002, Hassan *et al.* 2019). For nitrogen sources, ammonium sulfate

supported highest PHB production in KS-24 (52.5 mg/ml), while peptone supported maximum production in KS-25 (43.75 mg/ml) (Fig. 4B). Neutral pH (7.0) favored maximum production in both isolates (Fig. 5A), aligning with Shaaban and Mowafy (2012). Optimal temperature was 37°C, and maximum PHB yields were obtained after 72 hrs incubation (Fig. 5B-C). Longer incubation periods resulted in decreased yields, likely due to nutrient depletion or metabolic inhibition.

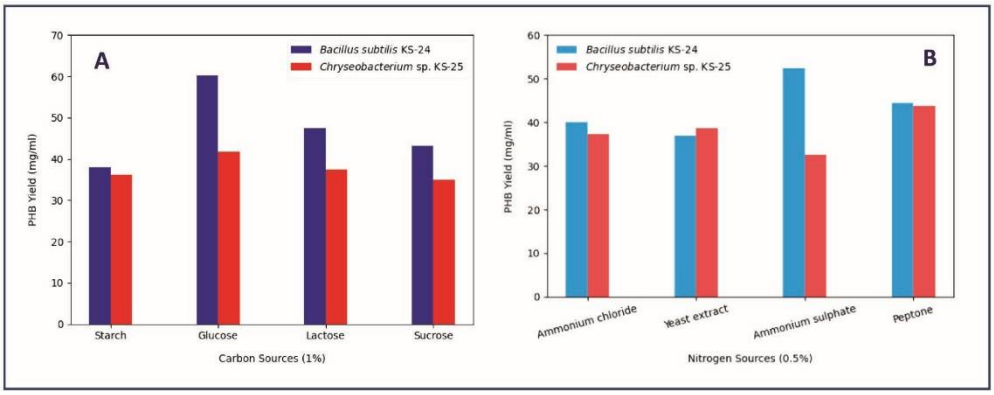

Fig. 4. Effects of nutrient sources on PHB production. A: Effects of different carbon sources, B: Effects of different nitrogen sources.

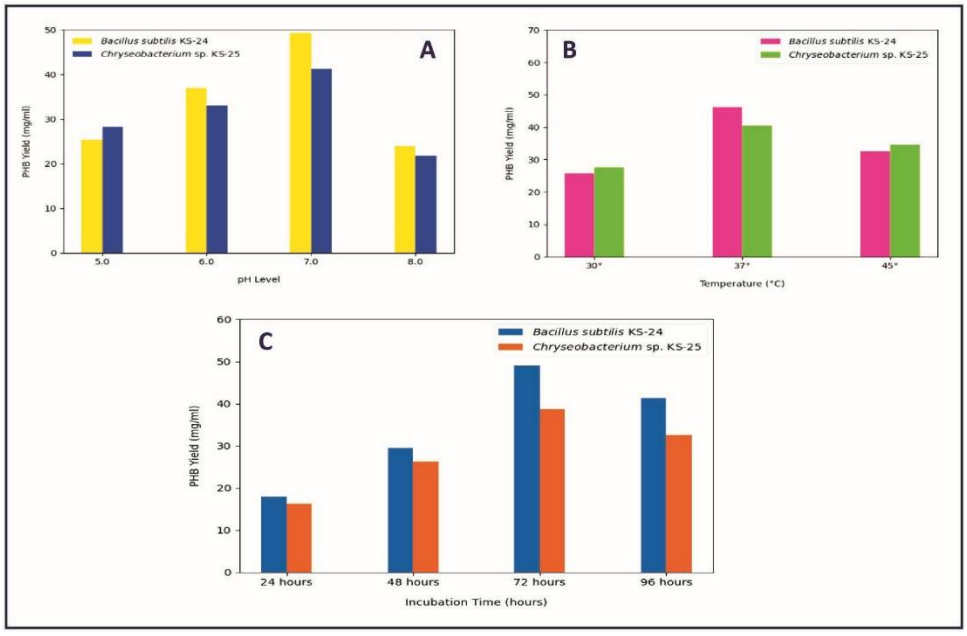

Fig. 5. Effects of physiochemical factors on PHB synthesis. A: pH, B: Temperatures, C: Incubation period.

Based on the findings of this study, *Bacillus subtilis* KS-24 and *Chryseobacterium* sp. KS-25 exhibit considerable potential for PHB production. The optimized culture conditions identified here offer valuable insights that can be applied to enhance PHB synthesis at an industrial scale.

However, due to the limited existing research on these specific strains, especially *Chryseobacterium* sp., further investigations are essential to fully assess their metabolic capabilities and scalability for commercial biopolymer production.

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