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Short Title: Isolation, screening and characterization of *Bacillus sp.* for whey based Polyhydroxyalkanoate (PHA) production

REVIEW ARTICLE

Isolation, screening and characterization of *Bacillus sp.* for whey based Polyhydroxyalkanoate (PHA) production

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Abstract

Whey is an excellent source of carbon for bacteria that produce *Polyhydroxyalkanoates* (PHA). There are no research employing raw whey, which has a very small amount of lactose, despite the fact that the majority of studies have utilized Whey, which includes large quantities of lactose as ingredient. Based on Sudan Black B colony staining, the following 30 bacterial strains were identified and chosen for further screening: S4, S8, S9, C10, C11, C12, C13, S15, S33, S35, and S37. The ideal circumstances for PHA synthesis will next be looked at, and the most effective isolate will be found using molecular characterization. 30 bacterial isolates were subjected to a two-step throughput screening procedure utilizing the lipophilic stain (Sudan black B) or fluorescence stain (Nile red) methods. Sudan Black and Nile Red, two separate indicator stains, will be used to examine bacterial isolates for PHA generation before the most productive isolate is found. On an increase in PHA production, which will be measured by converting PHA to crotonic acid, the research of variables influencing PHA production and stressful situations will alternatively rely. The biopolymer will be extracted using a variety of solvents. The current work offers a first exploration into finding and isolating a powerful Bacillus species that may produce PHA from dairy waste (whey). Helps handle dairy waste as solid waste, which reduces pollution and speeds up the recycling process.

Keywords: *Polyhydroxyalkanoates*, Sudan black B, Crystal violet, Nile Red-positive

Introduction

The most widely produced microbial plastics are Polyhydroxyalkanoates (PHAs) and their derivatives. Due to the use of petroleum plastics, we have been facing a number of issues leading to environmental pollution as plastics take years to degrade into soluble monomers which in turn release toxic gases. As a result of these challenges, there have been concerns regarding the development and utilization of biopolymers as a biologically valuable alternative for plastics (Vinet et al., 2011). Polyhydroxyalkanoates (PHA) have received significant consideration because of their qualities as biodegradable thermoplastics. Short chain length PHAs, medium chain length PHAs, and long chain length PHAs are the three categories for PHAs (mcl-PHAs, scl-PHAs, and lcl-PHAs respectively). These categories were broken down into scl-PHA, which had 6 carbon atoms-14 carbon atoms, which had 3 carbon atoms-5 carbon atoms, mcl-PHA, or lcl-PHA, which

had more than 15 carbon atoms, based on the number of carbon atoms (Bose et al., 2021; Bhattacharyya et al., 2012). PHAs are insoluble granules that can be generated by a wide range of microorganisms. They are able to accomplish this even in stressful environments with high carbon levels and low concentrations of oxygen, phosphorus, nitrogen. Gram-positive bacteria *Bacillus megaterium*is one among these microbes (Israni et al., 2020; Raho et al., 2020).

Bacillus subtilis, Pseudomonas, Alcaligenes eutrophus, Coryne bacterium glutamicum, or cyanobacteria like Synechococcus sp., Nostoc muscorum, and Spirulina platensis are examples of gram-negative bacteria.

Depending on the PHA manufacturer, the molecular weight of PHA ranges from 50,000 Da to 1,000,000 Da (Koller et al., 2017; Van Wegen et al., 1998). Of the various PHAs, Poly-3hydroxybutyrate (PHB) has the most understood structure among PHAs and *Bacillus sp.* the main interest group (Carletto et al., 2011). PHB is a thermoplastic material which has enhanced elongation and tensile strength.

Literature Review

Bhuwal et al. studied about numerous bacteria build up storage compounds called Polyhydroxyalkanoates (PHAs) as carbon and energy reserves. They are biocompatible bioplastics that are also biodegradable and ecologically friendly. Sudan black B, a preliminary screening tool for lipophilic substances, produced a black-blue coloring in 42 isolates, whereas Nile blue A, a more specialized dye for PHA granules, produced positive results in 15 isolates. The isolates NAP11 and NAC1 produced PHA at peaks of 79.27% or 77.63%, respectively, with waste water from the cardboard industry at polymer concentrations of 5.336 g/L or 4.042 g/L, respectively. Potential possibilities for industrial PHB generation include the strains *Enterococcus sp*. NAP11 or *Brevundimonas sp.* NAC1 from waste water from the cardboard industry (Bhuwal et al., 2013).

Joyline Mascarenhas and K. Aruna studied about screening of microorganisms that accumulate Polyhydroxyalkonates (PHA) from various environments the total number of isolates collected and cleaned up was 120. For the purpose of making bioplastics, Sudan Black B staining was employed to screen for cellular inclusions of lipids. Additionally, the positive lipid accumulators 34 cultures were examined for PHA buildup using the Nile blue a plate test. The aforementioned assay indicated PHA accumulation as positive. To encourage accumulation of isolates, sterile E2 medium was used to develop. The modified hypochlorite technique was used to extract PHA. They are PHA levels were monitored for maximal PHA generation by the approach of Slepecky and Law (Mascarenhas et al., 2017).

Danh H. Vu, et al. have investigated the feasibility of using Volatile Fatty Acids (VFAs) produced from food waste as low-cost carbon sources for the bacterial growth necessary to produce PHAs. It has been demonstrated that the inhibition phenomenon of propionic acid, or valeric acid while these volatile fatty acids were utilized as single carbon sources may be overcome by the use of VFA mixtures. By characterizing the isolated PHAs using techniques like FTIR and differential scanning calorimetry, it was established that they were Polyhydroxybutyrate (PHB). According to the research, VFAs generated during the acidogenic fermentation of food waste might be used as a cheap substrate to lower the price of making PHAs (Vu et al., 2021).

T. R. Elmashi et al. studied about the most powerful bacterial isolate generating Polyhydroxyalkanoates (PHAs) from industrial waste. The Arab Dairy Products Company collected 78 isolates from various locations. Optimized nutritional and physiological elements that affect PHA manufacturing. The optimal conditions for obtaining the maximum production of PHA 1.63 g/L with a recovery yield of 33.42 (%, w/w) after 96 hours were determined to be temperature 37 °C, whey content of 50%, and ammonium chloride 1.5g L. Utilizing FTIR spectroscopy, the properties of the isolated PHA were examined. The goal of this research was to increase *Bacillus cereus's* capacity to produce PHA from whey with a high level of purity a low-cost carbon source (Elmashi et al., 2022).

Micaela Pescuma et al. (Pescuma et al., 2015) However, innovative, affordable, functional whey-based foods and beverages have been developed, highlighting the health-improving qualities of fermented whey-derived goods by utilizing whey's high nutritional content. The numerous applications of whey as a resource raw material for the microbial fermentation-based manufacture of specific chemicals, meals, and drinks. The first study to summarize the process by which bacteria convert whey into a variety of foods and items with industrial utility.

Methodology

Design

This study design for the isolation and screening of *Bacillus sp.* for whey based Polyhydroxyalkanoate (PHA) production. To isolate Bacillus spp. from different environmental sources. And to screen the isolates for PHA production on Nutrient agar and Whey medium. To identify the potent isolate by 16S rDNA analysis. To determine the time, course PHA production by *Bacillus sp.* on whey medium.

Sample

The soil and curd samples that were collected from different sources and grown on Nutrient agar showed presence of different bacterial colonies which were further checked for their ability to grow on whey as a media. These colonies Figure 1 were morphologically different based on size, colour and shape. Among 30 bacterial strains isolated, based on Sudan Black B colony staining, the isolates that were selected for further screening processes were S4, S8, S9, C10, C11, C12, C13, S15, S33, S35, and S37.They were then further characterized morphologically, gram staining and for the PHB yield.

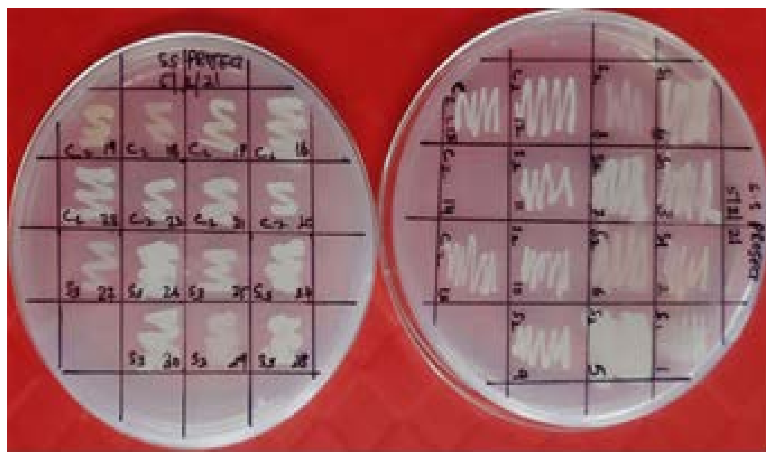

Figure 1. **Isolated cultures from soil and curd. The isolates also gave a positive growth when grown on whey media.**

Data collection

Isolation of PHA producing bacteria: Varied soil and curd samples from various biological niches were gathered in order to isolate PHA-producing bacteria. While the samples of curd were kept refrigerated until analysis, the soil samples were kept at ambient temperature. The dirt and curd samples were then successively diluted. In 9ml of sterile distilled water, 0.1gm of soil sample was added and serially diluted. A variety of dilutions of the curd sample were plated on nutritional agar (beef extract 0.3%, peptone 0.5%, sodium chloride 0.5%, glucose 1%, and agar 2%), after the curd sample was serially diluted by adding 1ml to 9ml of sterile distilled water.

Sudan black staining: In 1934, Lison developed Sudan black B as a particular fat stain for detecting lipids in tissue slices. Sudan black B is touted as being preferable to the other Sudan dyes since it stains bacterial fat bodies a deep blue-black hue when diluted in 70% alcohol or ethylene glycol (Hartman et al., 1940). Sudan Black B stain in an alcoholic solution with a concentration of 0.02% was used to quickly identify and isolate bacteria that produce PHB. The bacterial colony plates received the stain, which was then applied and left alone for 30 minutes. Extra stain was decanted, then 100% ethanol was used to wash it away. Due to the integration of the stain, the Sudan Black B positive colonies looked black, whereas the negative colonies were white.

Gram staining: Hans Christian Gram utilized the Gram stain for the first time in 1884. To characterize the phenotype of bacteria, the Gram stain is required. The staining process separates bacterial-related species into several groups based on the composition of their cell walls. Gram-positive cells stain from blue to purple and have a thick covering of peptidoglycan. Gram-negative cells with the number 31 have a thin peptidoglycan covering and colour from red to pink. To

understand colony shape and perform an initial organism screening, gram staining was used. On a slide, a thin layer of the organism was made. The excess stain was heat-fixed, decanted after 60 seconds of saturation with Crystal violet, and then Gram's iodine was added. The excess was emptied after 90 seconds. The slides were counter stained with Safranin after being ethanol washed for 5 seconds-10 seconds. After 45 seconds, distilled water was used to wash it. Under a microscope, the dyed cell slides were examined.

Rapid screening of PHB producing bacteria: Nile red screening**:** The Nile red staining was then used to screen the Sudan Black B positive isolates. By using a quicker, more sensitive, more viable colony, Nile red is a more accurate stain for PHA. There was a 0.5% concentration of dye applied (1 mg Nile Red was mixed in 1 ml acetone). To the Nutrient Agar medium, 50 l of this Nile Red solution were added. In the presence of dye, the plates were infected and then incubated for growth. The PHB-producing bacteria exhibit Bright Orange fluorescence when exposed to UV light, and the intensity of the fluorescence rises with the amount of PHB the bacteria are making. The Nile Red-positive isolates were then used to produce and extract PHB further.

PHB production on whey media: In the process of making cheese or casein from milk, whey, which accounts up 80%–90% of the initial volume of milk transformed, is a substantial by-product. It has a lactose content of 4.5% (w/v), a protein content of 0.8% (w/v), a salt content of 1.0% (w/v), and a lactic acid content of 0.1%-0.8% (w/v). After staining the positive isolates, they were cultured in Whey 32 medium. The Whey was obtained from Anand Sweets in Bangalore to guarantee that the quantity of sugar and protein were consistent. Sterile Whey broth in 100ml whey, monopotassium phosphate 0.2%, dipotassium phosphate 0.7%, magnesium sulphate0.01%, sodium citrate 0.05%, ammonium sulphate 0.1%, pH= 7) was inoculated with the select isolate inoculum (inoculum preparation: the loopful of selected culture organisms were inoculated in 10ml of sterile Nutrient Broth) and incubated in shaker incubator for 24hours.

Extraction of biomass: The incubated whey broths were then centrifuged at 8000rpm for 15 minutes (in duplicates). The media was decanted followed by washing of pellet twice with distilled water. The pellet was transferred to empty, pre-weighed eppendorf vials after being suspended in 1ml of distilled water. Centrifuged one more, then water was carefully decanted. The vials were kept open for drying to obtain dry cell mass which was taken as the biomass weight. The vials biomass was weighed until the weight was constant.

Extraction of PHB: The other set of inoculated whey broth were used for PHB extraction (in duplicates). The PHB was extracted from cells after the cells were lysed. 5ml of Sodium Hypochlorite were added to 10ml of whey broth for digestion of cell wall and release of PHB from the cells. The broth was kept for 2 hours of incubation in shaker incubator at 37°C. Following the incubation, the broth was centrifuged for 20 minutes at 10,000 rpm before the media was decanted. Two distilled water washes were performed on the white pellet. After that, the pellet was suspended in 10 ml of acetone and centrifuged once more for 15 minutes at 8000 rpm. Following centrifugation, the particle was placed in pre-weighed empty Eppendorf vials while being suspended in distilled water. The particle was centrifuged once more after which 1 ml of methanol was added. Once again centrifuged, the 33 suspension was saved for drying. The PHB was dried until the accumulated PHB weight remained constant.

Data analysis

Calculation of PHB percentage: The weight of biomass was taken and weight of PHB accumulated were taken.

To calculate PHB percentage

Weight of biomass= weight of dry cells - pre weight of vial

Weight of Accumulated PHB = weight of dry PHB – pre weight of the vial.

After the calculation of percentage of PHB production, the culture producing highest percentage was selected and subject for molecular identification of the organism.

Molecular identification of organism: Molecular characterization: The selected isolates, based on the dual property of efficient PHA and hydrolytic enzyme potential, were identified by 16S rRNA gene sequence analysis and phylogenetic studies. Using a genomic DNA extraction kit, the genomic DNA was extracted from the chosen isolates for this (Chromous Biotech India Pvt. Ltd., Bangalore). The universal primers listed below were used in PCR to amplify the isolates' partial 16S rRNA gene:

"Forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3'"
"Reverse Primer: 5'-ACGGCTACCTTGTTACGACTT-3'"

The PCR was initially denaturated for two minutes at 94°C before going through 40 cycles of denaturation at 34°C for one minute, annealing for one minute, and extension for 1.5 minutes. The most recent extension occurred at 72°C for 10 minutes. At Chromous Biotech India Pvt. Ltd. in Bangalore, the PCR product and a piece of the 16S rDNA sequence were purified. Using NCBI BLAST, a search for sequence similarity was conducted on the incomplete 16S rDNA sequence. The MEGA5 programme was then used to generate the phylogram from the multiple-alignment file. The building of a phylogenetic tree showing near homologs of the chosen isolates was carried out using neighbor-joining on a p distance matrix. In MEGA5, evolutionary analyses were carried out. The European Nucleotide Archive of the European Molecular Biology Laboratory received the 16S rRNA gene sequences for deposit (EMBL-ENA).

Time course optimization of PHB production: The identified organism was subjected to different incubation time in Whey broth media. Inoculum was prepared in sterile Nutrient Broth. The inoculum was inoculated in Whey broth maintaining previously mentioned conditions and incubated in shaker incubator for different time course (24h, 48h, 72h, and 96h). After the incubation period was over, extraction was biomass followed by extraction of accumulated PHB was done in order to compare the percentage production of PHB at different time course and also to estimate the lactose utilization during these time courses by the organism used. The time course where PHB production was highest was selected for further process.

Estimation of utilized lactose: The estimation is based on a titrametric approach using a modified Fehling's solution to determine the relative reduction abilities of lactose in the hydrolyzed and unhydrolyzed phases. Lactose content is determined by hydrolyzing lactose with beta-galactosidase, while sucrose concentration is determined by fructose 35's reducing activity as a result of that sugar's acid hydrolysis. 98.5% (91%-106%) of the lactose present is measured using the technique. The lactose was first estimated for unutilized lactose by directly using whey for titration. Burette was filled with Whey and in a flask equal volume (5 ml) of Fehling's solution A (7gm cupric sulphate was dissolved in 100ml of distilled water) and Fehling's solution B (35 gm of sodium potassium tartrate (Rochelle's salt) and 10gm sodium hydroxide was dissolved in 100 ml of distilled water) was added and titrated against whey. The flask with solution was boiled and titrated simultaneously against the filtrate till it reaches the end point (Blue to Brick red). The titration was repeated for concordant values. The titration was then done as per previously mentioned procedure for the utilised lactose by titrating Fehling's solution against broth media at different time course. The titration was repeated to obtain concordant values and a graph was plotted with concentration on X axis and Time course on Y axis.

Results and Discussion

Isolation and screening

A typical bacterial lipid polymer responsible for energy storage is Polyhydroxybutyrate (PHB). In nature, such as soil, ocean, sewage, or compost, there are a wide range of bacteria that may create PHB as a storage medium. Presently, it is known that over 150 distinct Hydroxyalkanoic acids are integrated into Polyhydroxyalkanoates, and it has been reported that microbial species from over 90 genera collect these polyesters. There have been reports of these bacteria in diverse habitats. Sudan black B or Nile blue 0.02% alcohol solution is used to quickly identify and isolate bacteria that produce PHB. A viable colony staining technique was employed.

Sudan black staining

Sudan Black is a somewhat basic dye that reacts with acidic groups in complex lipids to stain them as well. Since the PHB is a lipid-derived substance, it binds to Sudan Black and, when counter-stained with safranin, reveals dense black deposits inside cells.

Sudan Black B staining was performed for S4, S8, S9, C10, C11, C12, C13, S15, S33, S35, and S37 after a 24-hour culture period to confirm the presence of PHB granules. The goal of Sudan Black B stain is to make lipids visible. When the bacterial colony was dyed, the cytoplasm took on a pink hue, while the spores took on a bluish-black hue. Fig. 2 discusses the bacterial cultures that were positive for Sudan black B staining out of all the cultures tested. Positive results from the black staining process showed that they could all create PHB granules (fig. 2).

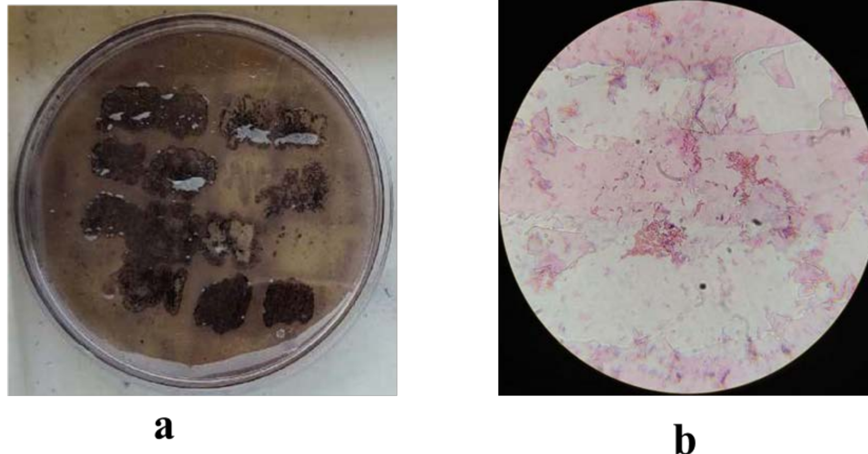

Figure 2. **A) Different isolates showing positive results for Sudan black staining. B) Sudan black staining showing bacterial cells (pink) with PHB granules (bluish black).**

Gram's staining

Bacillus and *Streptomyces*, two gram-positive bacteria, have been widely exploited in industry. These organisms, nevertheless, have not yet been used to make PHA biodegradable polymers. The sole commercial source of PHA at the moment, gram-negative bacteria include Lipopolysaccharides (LPS), which co-purify with the PHA and trigger immune responses. However, the absence of LPS in Gram-positive bacteria is a benefit that demands a thorough examination of their PHA synthesis. Due to its higher polymer yields but also less demanding fermentation conditions, the species *Bacillus* appears to be a suitable choice for the manufacture of PHB (Wu et al., 2020). Short Chain Length (scl) or Medium Chain Length (mcl) PHA may both be incorporated by the new PHA synthase from the *Bacillus* genus, showing that the genus has the capacity to create both novel and well-known PHA with a variety of monomeric compositions. As a result, the accumulation of PHB by the Bacillus genera has unique characteristics that require considerable research (Tu et al., 2018).

Gram staining is a differential staining method that helps identify various microbe types based on the components of their cell walls. These cells are dyed red or violet by gram staining to distinguish between Gram positive and Gram negative germs. Gram positive microscopic organisms stain violet because to the presence of a thick layer of peptidoglycan in their cell walls that protects the gem violet tone. Due to their thin peptidoglycan cell walls, which do not retain crystal violet when a decolorizing agent is added, Gram negative bacteria stain red (ethyl alcohol). All the strains were found to be Gram positive rods in nature fig. 3.

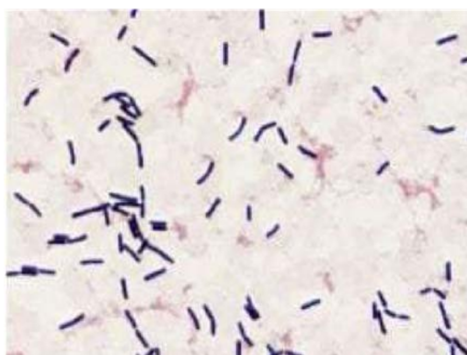

Figure 3. Gram positive bacilli.

NILE red staining

Nile Red is a lipophilic stain which in a lipid rich environment shows fluorescence deep red color. It is a fast and easy way to detect intracellular PHB which can be easily detected by fluorescence microscopy. Among 11 isolates screened for Nile red staining, fluorescence was observed in 5 bacteria tab. 1. which was because of binding of Nile red stain with PHB granules. So, the five bacterial cultures wiz, S35, S37, C10, C11, C13 were produced on a larger scale to estimate the percentage of PHB yield by all the positive bacterial cultures for Nile red.

Table 1. Staining results of different isolate from soil and curd.

Sample	Grams Staining	Sudan Black Staining	Nile Red
S_4	Gram positive	Positive	Negative
S_8	Gram positive	Positive	Negative
S_9	Gram positive	Positive	Negative
C_{10}	Gram positive short rods	Positive	Positive
C_{11}	Gram positive short rods	Positive	Positive
C_{12}	Gram positive very short rods	Positive	Negative
C_{13}	Gram positive long rods	Positive	Positive
$S1_5$	Gram positive rods with endospores	Positive	Negative
$S3_3$	Gram positive long rods	Positive	Negative
$S3_5$	Gram positive long rods	Positive	Positive
$S3_7$	Gram positive short rods	Positive	Positive

Determination of PHB yield

When carbon is abundant and nutrients like nitrogen, phosphorus, oxygen, or sulphur are scarce, certain bacteria, archea, and eukaryotes like yeasts and fungus create PHB as an energy reserve polymer. Lysing the cells is necessary to release the intracellular PHB granules since it is the storage molecule. This process of cell disruption is done by using the sodium hypochlorite solution. As PHB is soluble in various organic solvents, acetone and methanol are used to dissolve the PHB and evaporated till constant weights were obtained (Suzuki et al., 2022).

The percentage of PHB yield is calculated by measuring the extracted PHB weights and biomass of the product obtained after the fermentation for 48 hours in the shaker incubator. From the five strains which were selected after the successful screening for presence of PHB, its extraction was done. The PHB extracted from all the bacterial cultures was weighed to select the culture giving maximum amount of yield. The biomass and PHB produced by S_{35}, S_{37}, C_{10}, C_{11}, C_{13} are discussed in tab. 2. and according to it was determined that the bacteria C11 gave the highest yield of 88.3% PHB amongst all the other cultures.

More than 300 bacterial strains are known to be PHB producers, according to research, although there are many other strains that can create PHB compounds. Tab. 2. lists a few instances of strains that have been reported and used to generate. *Alcaligenes spp., Bacillus spp., Pseudomonas spp., Nocardia spp., Azotobacter spp., and Rhizobium spp.* are among the organisms that have been examined the most thoroughly, with *Ralstoniaeutropha* being the most well-known.

The greatest results for the formation of PHB, around 55.6%, came from the use of banana peels as a nitrogen source or sugarcane bagasse as a carbon source.

Table 2. **Biomass and Extracted PHB Weights of the samples which showed PHB and Nile red staining positive.**

Sample No	Biomass (g/L)	Extracted PHB (g/L)	Percentage of PHB yield
$S3_5$	2.13	0.23	10.80%
$S3_7$	1.47	1.2	81.63%
C_{10}	2.28	1.42	62.30%
C_{11}	1.72	1.52	88.30%
C_{13}	3.8	1.43	37.63%

Molecular identification

Molecular characterization of isolate C_{11} NCBI BLASTN analysis of the gene sequence of the isolate C_{11} identified the isolate as belonging to the genus Bacillus. The phylogenetic tree analysis revealed Bacillus subtilis strain to be the closest relative of isolate Bacillus subtilis MW343495 fig. 4. Hence the strain was designated as Bacillus subtilis C_{11}. The Gene-Bank accession no. for the 16S rRNA nucleotide sequence has been submitted.
Primer details
"Forward- 27F 5'-AGAGTTTGATCCTGGCTCAG-3'"
"Reverse- 1492R 5'-GGTTACCTTGTTACGACTT-3'"

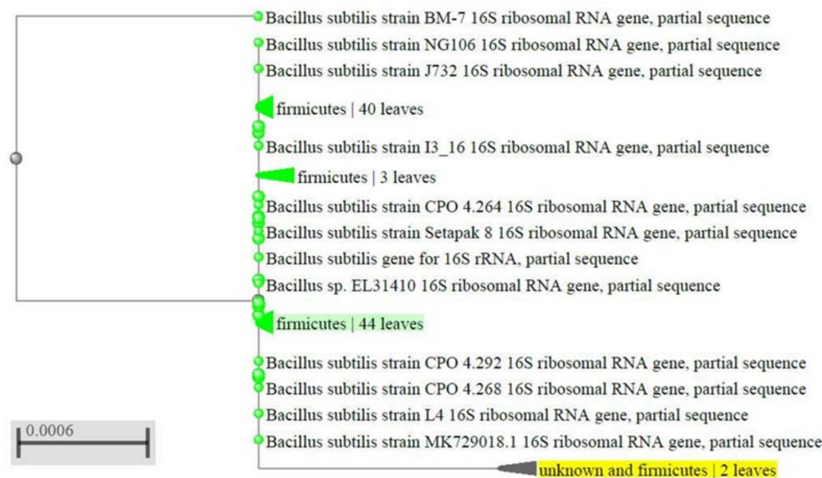

Figure 4. **Phylogenetic tree.**

Effect of incubation time on PHA production by bacillus Subtilis C_{11}:

The effect of incubation time was studied for the C11 strain which showed maximum PHB yield, in order to determine the optimum production time required for the maximum PHB production. The study was done at 24h interval till 96h, as shown in tab. 3. It was found that at 24h the biomass peaked with 1.95 g/L of PHB. However, later there was a dip in the biomass which remained steady after 48h with gradual increase in PHB yield peaking at 72h which remained steady till 96h of study.

Table 3. **Effect of incubation time on PHB production by *B. subtilis* C11.**

Time (in h)	Biomass (g/L)	Extracted PHB Weights (g/L)	Percentage of PHB yield
24	8.18	1.95	23.84%
48	4.2	2.99	71.19%
72	4.18	3.06	73.21%
96	4.14	2.8	67.63%

When cells are lacking a vital nutrient but have an abundance of carbon sources during the stationary phase of development, all bacteria that are able to synthesis PHB begin to accumulate it. Many Bacillus species have been shown to amass 9%-44.5% of their Dry Cell Weight (DCW) in PHA. PHAs' high production costs are a significant obstacle to their commercialization. By creating superior bacterial strains and effective methods for PHA fermentation and recovery, significant effort has been made to minimize PHA manufacturing costs. In order to lower production costs, providing renewable carbon substrates has been proposed as a practical and ecologically friendly option (Shimizu et al., 2020; Hadano et al., 2018). The cost of raw materials and energy consumption in the bacterial mixture of PHAs can be reduced by using leftover modern or urban trash, ranch excess, waste water treatment streams, or residual modern wastes as carbon substrates. Whey, which makes up 80%-90% of the original volume of milk altered, is a significant side effect of manufacturing cheddar or casein from milk. Its fat component is made up of 1.0% salts, 0.8% protein, 0.1%-0.8% lactic corrosive, and 4.5% (w/v) lactose. Due to its high requirement for organic oxygen, only about 50% of the whey product may be used for animal feed; the remaining portion is wasted, which has a negative impact on the environment. As a result, several safe microbial maturation-based whey usage processes have been developed.

Lactose estimation

To assess the quantity of lactose in the whey that is used by the organism for growth, lactose was determined by titration with Fehling's reagent. Blue $Cu2^+$ ions will be converted to Cu^+ ions by aldehyde containing sugars, but under normal circumstances, $Cu2^+$ prefers to precipitate as an insoluble compound with hydroxide ions. In order to stop the precipitation, tartrate ions are added to the solution, which separates $Cu2^+$ from hydroxide by forming a soluble complex with $Cu2^+$. The equivalence point of the reaction is shown by the disappearance of the deep blue copper (II) tartrate complex and the emergence of the Cu+ ions as a red precipitate. When the amount of lactose used by the organism was determined, it was discovered that the maximum amount was used after 24 hours, during which time biomass was formed and more lactose was converted into PHB. The lactose consumption was discovered to remain consistent at 48 hours, 72 hours, and 96 hours, indicating that the organism has fully consumed all lactose within 48 hours.

Conclusions

The current work offers a first exploration into finding and isolating a powerful Bacillus species that may produce PHA from dairy waste (whey). The study also supports the idea that adding whole whey as a carbon source to the production medium can improve the output of PHB. Additionally, it offers a practical method for producing PHB at a cheap cost and helps with the recycling process and solid waste management of dairy waste.

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