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Production of Polyhydroxyalkanoate (PHA) by a Moderately Halotolerant Bacterium *Klebsiella pneumoniae* U1 Isolated from Rubber Plantation Area

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Abstract Polyhydroxyalkanoates (PHAs) are polymers which are produced and degraded by many strains of bacteria. They have properties similar to polypropylene, which is a commonly used petrochemical-based plastic. In the present study, five different bacterial strains were isolated from rubber plantation area, of which only one isolate (U1) was able to produce PHA. Screening for PHA was done by Sudan Black B staining and Nile Blue A staining. The strain U1 produced PHA when cultivated in media with different carbon and nitrogen sources, and the optimal carbon-nitrogen source combinations for PHA production was found. PHA extraction was carried out by sodium hypochlorite digestion method. 16S rRNA analysis showed that the PHA-producing bacterium had maximum sequence similarity with *Klebsiella pneumoniae*. PHA was analysed by UV spectrophotometry and characteristic functional groups were identified by FTIR spectroscopy. In this study, *Klebsiella pneumoniae* has shown an ability to produce PHAs using different substrates in acidic and halophilic conditions. Therefore, by optimising the process of production, *Klebsiella pneumoniae* can be used to produce bioplastics in commercially significant amounts.

***Keywords**: Polyhydroxyalkonate (PHA), moderately halotolerant, bacterial strain, bioplastics, biopolymer*

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1. Introduction

In the society of today, plastics are extensively used as they are durable due to their thermal and mechanical properties. They are also easy and inexpensive to produce and process. They can be moulded into any shape, and are therefore rapidly replacing metals, glass and wood in various applications [1]. These petrochemical-based plastics cause pollution due to their non-biodegradable nature. Increased awareness about the harm caused to the environment, depletion of coal and natural gas reserves, increasing price of crude oil and global warming among other things have induced industries and institutes to research more cost-effective methods for production of environmentally-compatible alternatives to petrochemical-based plastics [2]. Polyhydroxyalkanoates are a class of linear polyesters which possess properties comparable to petrochemical-based plastics [3], with the additional advantages of being biocompatible and biodegradable. They are of interest since they can be produced and degraded by microorganisms. Poly-3-hydroxybutyrate (PHB or P3HB) is the most commonly known and researched PHA [3,4].

PHAs are of commercial interest as they can be processed into bioplastics, an environmentally-friendly alternative to the conventional petrochemical-derived plastics. They have promising applications in medicine, material science and agriculture [5]. PHAs are used in food packaging, plastic films, surgical sutures, controlled drug delivery, bone plates, nails, screws, and in the treatment of Osteomyelitis [6]. They can be derived from renewable and inexpensive sources, and are completely biodegradable by many microorganisms. In order to optimize costs, different bacterial strains are being studied for efficient PHA production using various waste materials and industrial effluents as renewable and inexpensive substrates. Recombinant strains are also being studied for improved PHA production [7].

Some microorganisms which are moderately or extremely halotolerant may have the ability to produce PHAs. These PHA-producing microorganisms can be isolated and put to use in the treatment of effluents which have a similar salt concentration to the environment in which they flourish [8]. Also, high salt concentrations prevent growth of other mesophilic organisms, so there is no need for strict sterile conditions and repeated sterilization of the equipment or media used. With such organisms, a dual purpose of effluent treatment as well as cost-effective production processes due to reduction in the use of energy can be achieved [9]. The reduction in cost will be greatly beneficial to mankind and will encourage the manufacture and sale of biocompatible plastics. The

increased use of environmentally-friendly products will lead to a cleaner and greener world in which all organisms can live healthier lives. In this present study, PHA producing bacterial strains was enriched from soil of a rubber plantation area. The growth parameters for PHA production by the bacterial strain were optimized under various carbon and nitrogen sources. The product produced by the bacterial strain was confirmed for presence of PHA by UV Spectrophotometry and FTIR Analysis.

2. Materials and Methods

2.1. Sample Collection

Soil samples were collected from rubber plantation area in Wayanad, Kerala. Soil samples were collected in sterile zip lock covers and transferred immediately to the laboratory and stored in a refrigerator at 4°C.

2.2. Isolation of PHA-Producing Bacterial Strains

1 gram of soil sample collected was inoculated in 100 ml Nitrogen-deficient medium having the following composition (g/L): Yeast extract (1.5), Ammonium nitrate (0.286), Potassium dihydrogen orthophosphate (0.75), Calcium chloride (0.4), Magnesium sulphate (0.4), Glucose (40) and pH was adjusted to 4. After 24 hour intervals, serial dilutions were made and the dilutions 10^{-5}, 10^{-6} and 10^{-7} were plated on Nitrogen-deficient medium containing Bacteriological agar (15 g/L).

2.3. Screening for PHA Production

Bacterial isolates were screened for PHA production by Sudan Black B and Nile Blue A Staining procedures.

2.3.1. Sudan Black B Staining

Heat-fixed bacterial smears were stained with 0.3% (w/v) Sudan Black B in 60% Ethanol for 10 minutes, rinsed, and counter stained with Safranin. The slide was air dried and observed under 100 X oil immersion [11].

2.3.2. Nile Blue A Staining

Heat-fixed bacterial smears were stained with 1% (w/v) aqueous Nile Blue A Stain and heated in a water bath at 55°C for 10 minutes. The stain was washed off with 8% (v/v) aqueous solution of acetic acid for 1 minute. The slide was allowed to dry and then viewed under fluorescence microscope at 460 nm excitation wavelength [12].

2.4. Extraction of PHA

PHA produced was extracted according to a modified version of the protocol followed by [13]. 10 ml of the culture broth was centrifuged at 10,000 rpm for 10 minutes to harvest the cells. To the culture pellet, 10 ml of sodium hypochlorite was added and centrifuged at 5,000 rpm for 20 minutes. The pellet was washed sequentially with distilled water, acetone and methanol, respectively. The pellet obtained after washing was suspended in 5 ml of chloroform, and evaporated in a glass Petri dish at room temperature. The weight of the residue obtained after evaporation of chloroform was recorded.

2.5. Analysis of Product

The extract was analysed for presence of PHAs using the UV Spectrophotometric method of [14]. Presence of PHA was further confirmed by FTIR analysis of functional groups.

2.5.1. Analysis of Product by UV Spectrophotometry

The presence of PHAs extracted from the bacterial isolate was analysed by UV Spectrophotometric method postulated by Law and Slepecky (1960). This method of confirmation uses the principle that PHAs are converted to crotonic acid when heated at 100°C with concentrated sulphuric acid for 10 minutes. Crotonic acid gives a maximum absorption peak at 235 nm. Hitachi U-2000 Spectrophotometer was used in this method of analysis. Equal weights of the extract (test) and crotonic acid crystals (standard) were heated with concentrated sulphuric acid at 100°C in two test tubes. Absorption maxima of the Test and Standard were found by performing a wavelength scan and the results were compared.

2.5.2. Fourier-Transform Infra-Red Spectroscopy Analysis

FTIR analysis of the product was carried out in a Perkin Elmer Spectrum1 FTIR instrument at Sophisticated Analytical Instrument Facility, IIT Madras, in the IR range of 4000 to 450 cm^{-1}. Peak values obtained were used to interpret the presence of specific functional groups in the extract.

2.6. Determination of Protein Content Using Bradford’s Method

Total protein content of the isolated bacterial strain was analysed using Bradford’s Method of Protein Estimation. Bovine Serum Albumin was used as standard protein solution. Standard curve was plotted using the standard protein absorbance against concentration. The protein content in the sample was calculated using the standard curve. Along with the protein estimation for each day, extraction of PHA was performed to determine the point in the growth curve of the bacterial strain at which yield of PHA is the highest [15].

2.7. Optimisation of Growth and PHA Production by Varying Carbon and Nitrogen Sources

Growth by protein estimation and yield of PHA were checked after varying carbon and nitrogen sources. Carbon sources such as Glucose, Glycerol, Sunflower oil and Sucrose were used in the study. Nitrogen sources like Ammonium Nitrate, Ammonium Chloride, Peptone and Sodium Nitrite were used. Carbon sources were added at 4% and nitrogen sources were added at 0.0286% in the mineral salts medium.

2.8. Screening for Halotolerance

To the study the level of halotolerance of the isolated bacterial strain, it was inoculated in nitrogen-deficient broth containing varying concentrations of sodium chloride (0%, 5%, 7% and 10%), and growth and protein yield were studied.

2.9. Biochemical Characterisation

The bacterial strain isolated was studied for morphological and biochemical characteristics. Initially, Gram staining and motility test were performed after which biochemical characterization was done (with Himedia, India) to identify the phenotypic characters of the bacterial strains. Catalase, Oxidase, Urease, IMViC and Triple Sugar Iron Agar Tests were performed to determine the biochemical characteristics of the isolate. After 24 h incubation at 37°C, the colour change observed was determined as positive/negative result. Genus level identification of the unknown bacterial strain was accomplished by using Bergey's Manual of Systematic Bacteriology (2005) [16].

2.10. 16S rRNA Partial Gene Sequencing

Chromosomal DNA was isolated from the pure strain by the standard phenol/chloroform extraction method [17]. The 1.2 kilo base partial sequence of the 16S rRNA gene was amplified from the chromosomal DNA using polymerase chain reaction (PCR) with universal Eubacteria-specific primers 16F27 (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16R1525XP (5'-TTCTGCAGT CTA GAA GGA GGT GWT CCA GCC-3') [18]. The PCR conditions used were an initial denaturation at 94°C for two minutes, followed by 35 cycles of denaturation at 95°C for one minute, annealing at 55°C for one minute, and extension at 72°C for one minute, and a final extension at 72°C for 10 minutes and sequenced on an ABI310 automated DNA sequencer using the Big Dye terminator kit (Applied Biosystems 3730 x DNA Analyzer). The amplified 16S rRNA gene PCR products from these isolates were directly sequenced after purification by precipitation with polyethylene glycol and NaCl. The primers used to obtain the complete sequence of 16S rRNA gene of the isolates were the same as for PCR amplification (16F27N and 16R1525XP).

Sequence data analysis was done using ChromasPro and Sequencing Analysis software. Further, phylogenetic tree was constructed using MEGA6. The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 0.04720271 is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 694 positions in the final dataset. Evolutionary analyses were conducted using MEGA6 [19].

3. Results and Discussion

3.1. Enrichment and Isolation of PHA Producing Bacterial Isolate

Five bacterial strains were isolated from the soil of rubber plantation area in mineral salts medium. Incubation of the soil sample in Nitrogen-deficient Broth and subsequent plating on Nitrogen-deficient Agar showed that only one bacterial strain (U1) was able to survive in the conditions of the medium (Figure 1). This isolate was found to be positive for intracellular granules stained by Sudan Black B stain and Nile Blue A fluorescent stain, which indicated PHA accumulation in the bacterial cell cytoplasm. Gram's staining and morphological and biochemical analyses revealed that the PHA-accumulating strain belonged to the genus *Klebsiella*. 16S rRNA analysis and BLAST analysis showed that the sequence had maximum identity (98%) with the 16S rRNA sequence of *Klebsiella pneumoniae subsp. pneumoniae* MGH 78578 strain ATCC 700721.

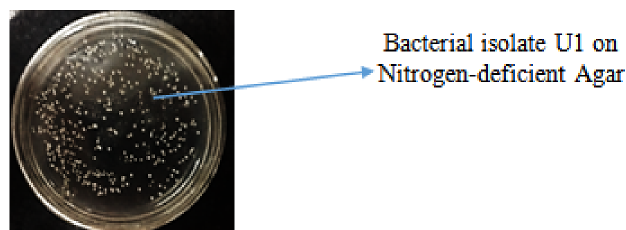

Figure 1. Enriched Bacterial isolate (U1) on Nitrogen-deficient Medium

3.2. Screening for PHA Production from the Bacterial Isolate

PHA granules accumulated within the bacterial strain U1 was visually confirmed by Sudan Black B staining where the PHA granules were darkly stained (Figure 2). This morphological observation of the intracellular granules proved the presence of PHA. Figure 3 shows that the Nile Blue A stain imparted a bright orange fluorescence to the PHA granules which was observed at a wavelength of 460 nm.

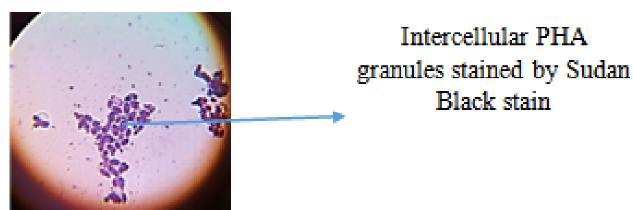

Figure 2. PHA granules in Bacterial strain U1 by Sudan Black Staining

Table 1. Weight of PHA in mg extracted over 4 days

Day	Weight (mg/ mL)
1	0.4
2	0.6
3	0.2
4	0.2

3.3. Extraction of PHA from the Bacterial Isolate U1

PHA produced by the bacterial strain U1 was extracted in powder form by lysing the cells with sodium hypochlorite over a period of 4 days (Figure 4). The weight of extracted PHA obtained on each day in mg is tabulated in Table 1. In the table, it is shown that there was an increase in PHA produced to 0.6 mg/mL or 0.6 g/L on Day 2. [21] studied PHA production in 11 different

Bacillus spp. and found PHB consisting 50% (w/v) of dry cell weight of the bacteria.

PHA production by the bacterial strain U1 is show in the Figure 5, which indicated that PHA is a growth-associated product, and its accumulation significantly increased when the culture reached stationary phase (about 24 to 48 hours). The maximum values were achieved at 48 h cultivation. After 48 hours, a sharp decrease in the level of PHA content was noticed. This indicated the presence of an intracellular PHA depolymerase. Concentration of PHA decreased significantly after 72 hours cultivation due to nutrient depletion and consumption of PHA as a carbon source. PHA production was maximum after 48 hours of incubation showing 0.6 mg/mL (6 g/L). The yield decreased during decline phase to 0.2 mg/mL (0.2 g/L) (Figure 5a). Maximum growth was observed after 24 hours after which there was a decline in protein content (Figure 5b), which indicated a decrease in protein formation due to depleted nitrogen source. The lowered total protein content of the bacteria corresponded with high amount of PHA production within 48 h cultivation. These results were similar to those given by *Ralstonia eutropha* (recent name *Cupriavidus eutropha*), which accumulated PHB at the stationary phase [21].

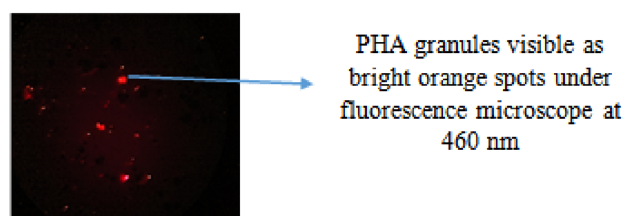

Figure 3. PHA granules observed as bright orange granules at 460 nm

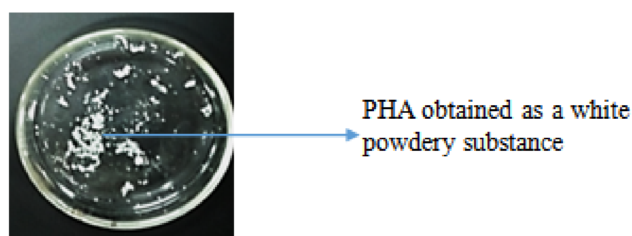

Figure 4. Extracted PHA after purification and drying

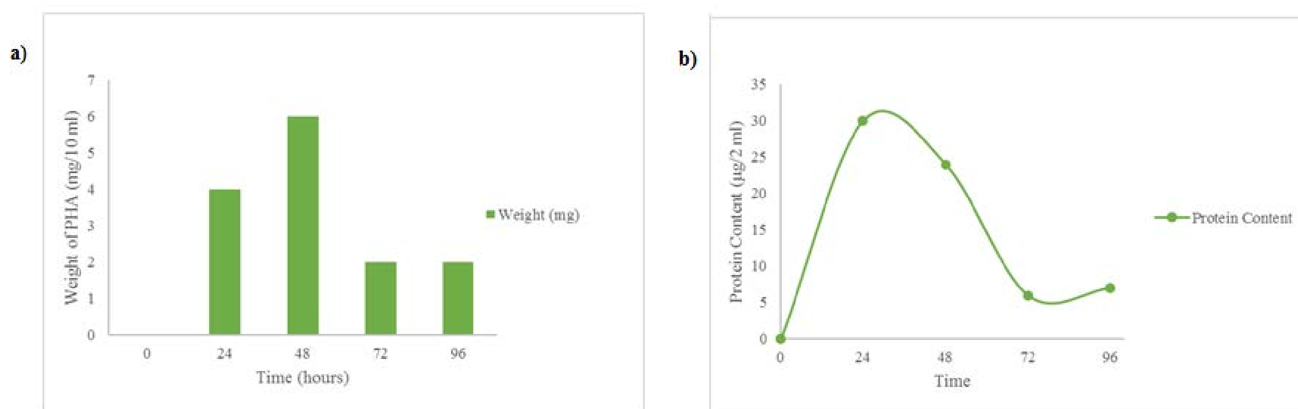

Figure 5. (a) PHA produced by the strain U1, (b) Total Protein content of the strain U1

3.4. Analysis of the Extract

3.4.1. UV Spectrophotometric Analysis of PHA Produced by the Bacterial Isolate U1

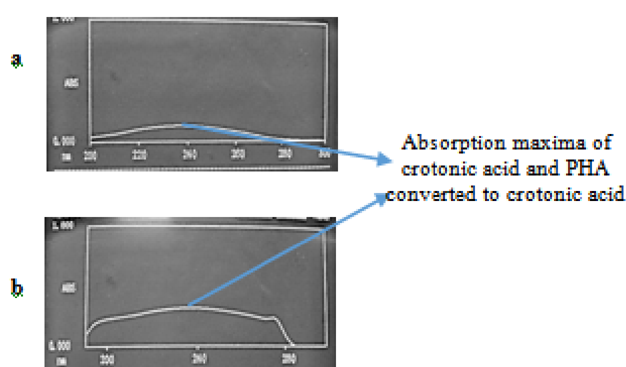

Figure 6. UV Spectrum -Absorption maxima of a) Reference Crotonic acid (Standard) b) PHA converted to crotonic acid (Test)

The PHA produced by the bacterial strain U1 was analysed by UV Spectrophotometry [14]. From Figure 6b, it was observed that the powder extracted from the bacterium showed maximum absorption at ~235 nm. The crotonic acid used as a standard in this analysis also showed maximum absorption at around the same wavelength (Figure 6a). From this it is possible to convey that PHA present in the extract has been converted to crotonic acid, and therefore it can be proved that PHA is produced by the bacterial isolate.

3.4.2. Fourier-Transform Infra-Red Spectroscopic Analysis

To further confirm the production of PHA by the bacterial isolate, powdered PHA samples were analysed by FTIR to determine if the characteristic functional groups of PHA are present in the extract. The peaks showing presence of ester group, methylene group and terminal hydroxyl group which are found in the polymeric structure of PHAs are depicted in Figure 7. From the figure, the characteristic peaks observed were 3408 cm^{-1} (terminal OH group), 2923 cm^{-1} (methylene C-H group) and 1745 cm^{-1} (ester carbonyl C=O group) [7]. Hence, the FTIR analysis confirmed the presence of PHA in the extract by showing the presence of characteristic functional groups of PHAs.

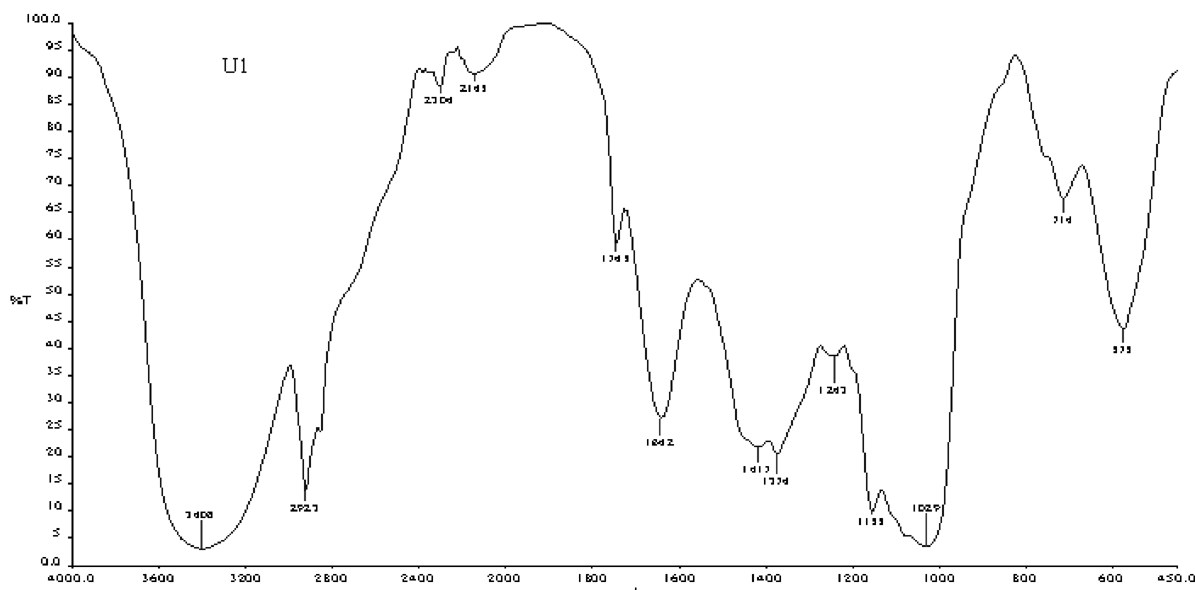

Figure 7. FTIR chart showing characteristic peaks of PHA produced by the isolate U1

3.5. Optimisation of Growth and PHA Production of the Isolate U1

3.5.1. Optimisation by Varying Carbon Sources

The main nutrient required for PHA production by bacteria is an excess amount of carbon source in the medium. From Figure 8a, it was observed that with glycerol as carbon source, strain U1 gave the highest yield of PHA (0.9 mg/mL or 0.9 g/L) after 48 hours of growth with ammonium nitrate as nitrogen source. During that time, protein content in the sample was 90.1 µg/ml (Figure 8b). The lowest yield of PHA was given by the strain U1 with sunflower oil as the carbon source (0.1 mg/mL or 0.1 g/L).

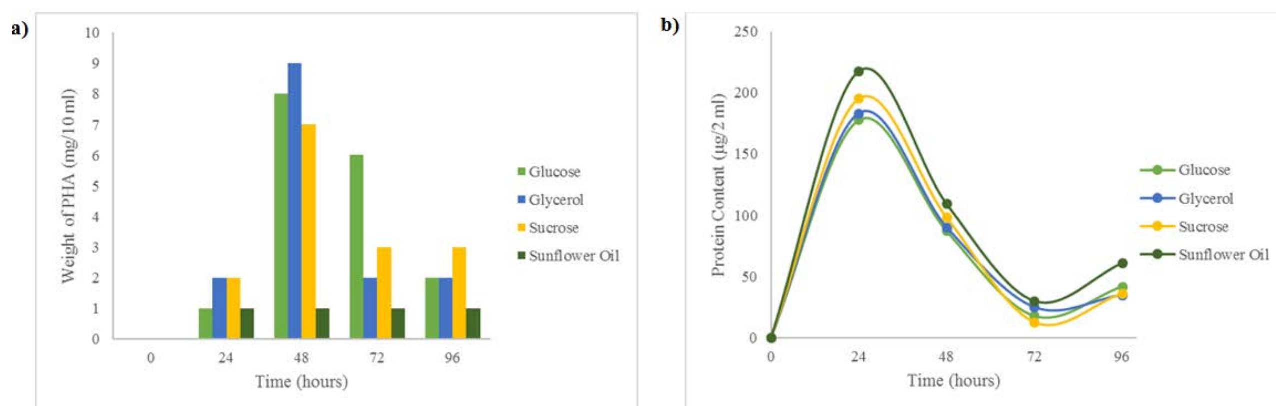

Figure 8. a) PHA production by the isolated strain U1, b) Total Protein content of strain U1 in various carbon sources

Chaijamrus and Udpuay (2008) have reported a PHA production of 7 g/L by *Bacillus megaterium* using molasses as carbon source. This shows that unrefined carbon sources obtain better yields as compared to refined sugars. Another study by Prasanna *et al* in 2011 yielded 0.93 g/L by *Bacillus megaterium* with glucose as carbon source. *Rhodobacter sphaeroides* was able to utilise acetate and yielded a maximum PHA production of 6.53 g/L after 60 hours of growth [3]. Using wastes from cardboard and paper industry as carbon source, *Enterococcus sp.* and *Brevundimonas sp.* were able to accumulate PHA to concentrations of 5.236 g/L and 4.042 g/L, respectively, after 72 hours of growth [24].

3.5.2. Optimisation by Varying Nitrogen Sources

From Figure 9a, it was observed that both ammonium chloride and peptone as nitrogen sources gave high yield of PHA (1.1 mg/mL or 1.1 g/L) individually after 48 hours of growth with glucose as carbon source. During that time, protein content in the sample was 89.5 µg/ mL for ammonium chloride and 85.9 µg/ mL for peptone (Figure 9b). The lowest yield of PHA was given by the isolate U1 with ammonium nitrate as nitrogen source (0.8 mg/mL or 0.8 g/L). Sangkharak and Prasertsan (2008) reported that lower concentrations of nitrogen sources gave optimal results of PHA yield. The effect of nitrogen sources are dependent on the type and concentration of carbon sources present in the medium for PHA production.

Thus, the combinations of glucose and peptone and glucose and ammonium chloride yielded the highest concentrations of PHA from the isolated strain U1 at 1.1 g/L, followed by glucose and sodium nitrite at 1 g/L after 48 hours of growth.

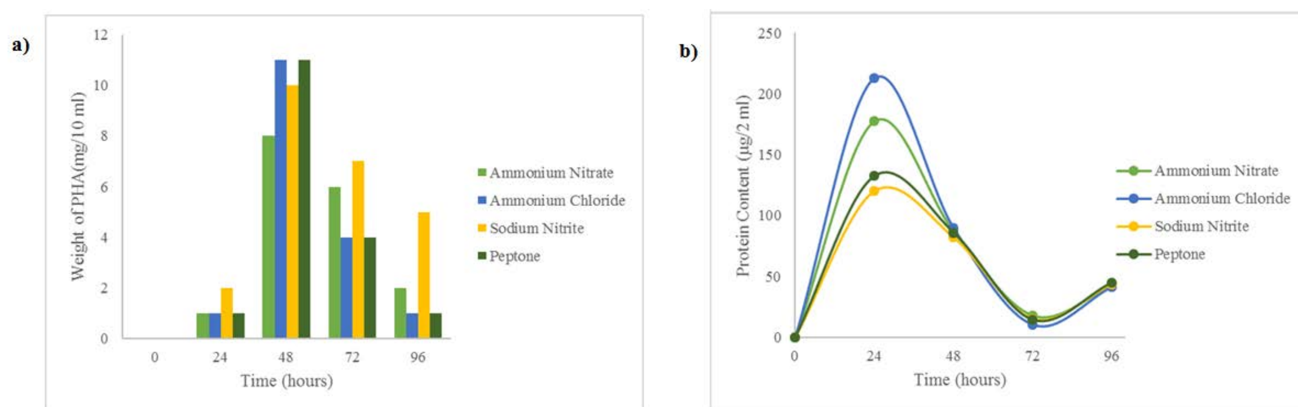

Figure 9. a) PHA production by the bacterial strain U1, b) Total Protein content of isolate U1 in various nitrogen sources

3.6. Screening for Halotolerance

Isolated strain U1 was screened for halotolerance by inoculating it in Nitrogen deficient medium containing NaCl in concentrations of 0%, 5%, 7% and 10%. From Figure 10a, it was observed that 0% NaCl concentration gave the maximum yield of 1 g/L after 48 hours of growth. From Figure 10b it was concluded that the isolate U1 was able to survive in NaCl concentrations of up to 10%, making it moderately halotolerant. However, it is able to efficiently produce PHAs in NaCl concentrations of only up to 5%, as seen by the average yield over 4 days (Figure 11).

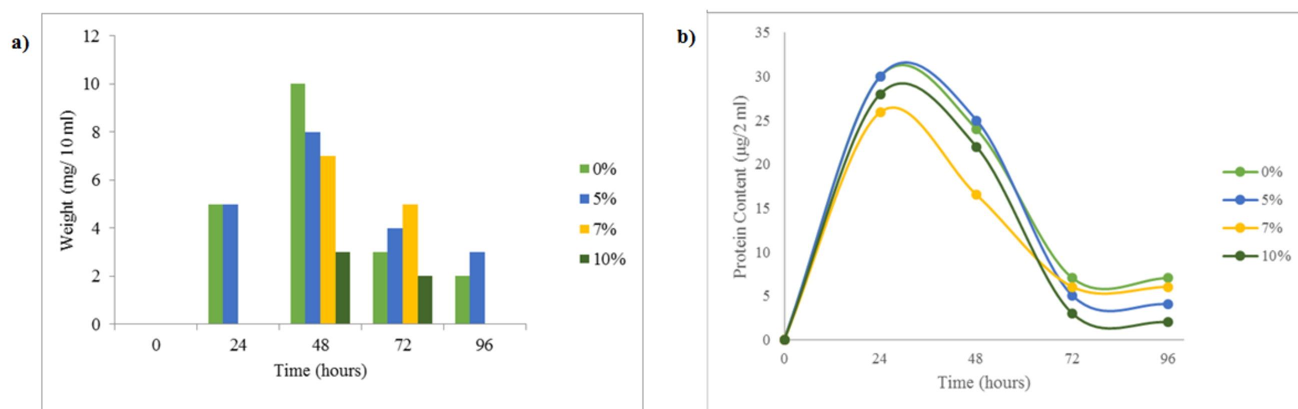

Figure 10. a) PHA production by the strain U1, b) Protein content of strain U1 at different NaCl concentrations

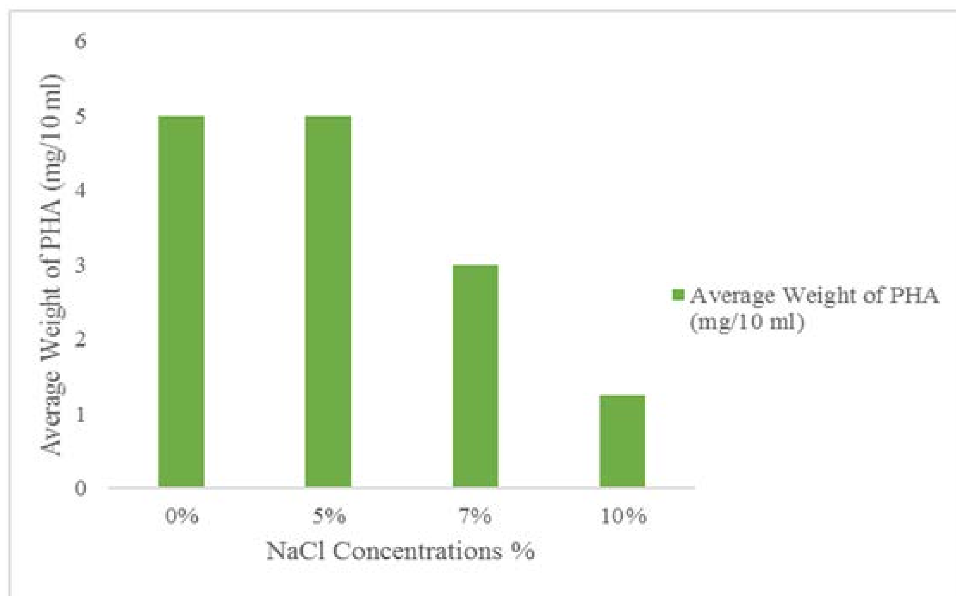

Figure 11. Average yield of PHA by the isolate U1 in various NaCl concentrations

3.7. Morphological and Biochemical Characterisation

Table 2 summarises the results of the morphological and biochemical tests performed on the isolated strain. The strain formed white, smooth, round mucoid colonies

on nutrient agar plate. Through 16S rRNA sequencing, the strain U1 was identified as *Klebsiella pneumoniae subsp. pneumoniae* MGH 78578 strain ATCC 700721.

Table 2. Morphological and Biochemical characteristics of bacterial isolate U1

CHARACTERISTICS	U1
Gram Staining	+
Cell Shape	Rod
Motility	-
Oxidase	-
Catalase	+
Urease	+
Indole	-
Methyl Red	+
Voges-Proskauer	-
Citrate Utilisation	+
Triple Sugar-Iron Agar	Acid butt, Acid slant

3.8. Identification of the Isolated Bacterial Strain by Phylogenetic Analysis

3.8.1. Description of *Klebsiella pneumoniae subsp. pneumoniae* strain MGH 78578:

BLAST analysis of nucleotide sequences from U1 strain showed a maximum of 98% identity towards *Klebsiella pneumoniae subsp. pneumoniae* strain MGH 78578. Hence, U1 was identified as *Klebsiella pneumoniae subsp. pneumoniae.* The strain U1 belongs to Super kingdom: Bacteria, Phylum: Proteobacteria, Class: Gammaproteobacteria, Order: Enterobacteriales, Family: Enterobacteriaceae, Genus: *Klebsiella*. The bacterial strain belonging to the phylum Proteobacteria is a non-motile, Gram negative, rod-shaped bacterium that is found in the normal flora of the body. It also occurs naturally in the soil. According to the biochemical tests, this strain showed positive results for Catalase, Methyl Red, Citrate Utilisation and Urease tests, and negative results for Oxidase, Indole, Voges-Proskauer tests. Figure 12 shows the Neighbour-Joining tree for strain U1.

Figure 12. Phylogenetic analysis of *Klebsiella pneumoniae subsp. pneumoniae* strain MGH78578 (U1) with its nearby substrains. The numbers at the nodes represent bootstrap values (500 replicates)

4. Conclusion

Among five bacterial strains isolated from the rubber plantation area, only one bacterial strain *Klebsiella pneumoniae* U1 which was identified with 16S rRNA sequencing alone produced polyhydroxyalkanoate and this was confirmed by FTIR analysis. This bacterial strain gave the highest value of growth with PHA production in salt concentration of up to 5% which showed the halotolerant nature of the isolate. *Klebsiella pneumoniae* U1 species isolated from the soil samples of rubber plantation area can be employed in the industrial production of PHA with further optimisation of culture conditions.

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