Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 04 | 2023
DOI 10.17605/OSF.IO/6AMUQ

SYNTHESIS OF POLYHYDROXYALKANOATES (PHAS) UTILIZING MOLASSES AS CARBON SOURCE BY PROTEUS MIRABILIS

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Abstract

Polyhydroxyalkanoates (PHAs) are produced by many bacteria & can be used as an alternative for the traditional petrochemical based plastics. The biodegradability & biocompatibility are the properties drawing attention towards use of PHA as bioplastics in the recent years. The aim of this study was to produce PHA by the novel bacterial strain isolated from the oil contaminated soil samples, the inexpensive carbon sources used were glucose & molasses. The bacteria were isolated, confirmed for positive PHA accumulation by Nile blue staining & Sudan Black B staining. The novel positive PHA producer Proteus mirabilis was emphasized in this study to check the growth pattern after 30 hours cultivation giving the maximum yield of 13.02% (using glucose as a carbon source) & 25.49% (using molasses as a carbon source) at optimum conditions pH 7.0 and a temperature of 35-37°C. The structure & functional groups of PHA granules extracted from Proteus mirabilis were analyzed using Fourier transform infrared spectroscopy (FTIR). Hence, Proteus mirabilis is the bacterial strain identified that can be used for the production of PHA giving lower to moderate quantities of PHA in this study.

Keywords

Bacteria, FTIR, Molasses, Nile blue Staining, Polyhydroalkanoates, and Proteus Mirabilis

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1. INTRODUCTION

Plastic materials are presently regarded as extremely essential due to their characteristics and superior performance to other materials like metal and wood. At present, the estimate of annual global output of consumer plastics is more than 300 million tons. Since plastics are employed in so many different applications, the problems that are directly or indirectly related to the environment and economy have been elevated (Alvarez-Chavez et al., 2012).

Some other environmental problems include the rise in the plastic waste in oceans, the release of toxic gases during plastic incineration and the non-degradability of plastic. Environment-friendly materials, such as bioplastics, have been developed & used in recent years as a result of these social, economic, and environmental issues. In the 21st century, the development of bioplastics will bring significant impact in the field of materials, in future the production of bioplastics will rise at the global level (Arikan & Ozsoy, 2015).

Bioplastics are biodegradable plastics that differ from petroleum-based plastics. The petroleum-based plastics such as polymer are unable to degrade and are causing serious environmental issues. Bioplastics, being ecofriendly and biocompatible originated from living organisms such as bacteria, therefore, can be degraded easily after use. (Peelman et al., 2013)

Bioplastics are produced from renewable materials that may be recycled organically through biological processes. In general, "bioplastic" is created by a variety of microorganisms from renewable resources like maize, sugar, potatoes, etc (Karana, 2012). The biodegradable plastic can be degraded easily by any commonly and naturally occurring microorganisms such as fungi, bacteria, and algae, the speed of this degradation process is affected by the environmental factors such as water, temperature and oxygen as well as the chemical state of the polymer. The very low CO2 emission during bioplastic breakdown highlights the need for the daily manufacture of biodegradable plastic (Scaffaro et al., 2016).

Throughout the world, governments have established a number of regulations to minimize the use & consumption of non-biodegradable plastics, several research groups have also worked to create biodegradable polymers including starch-based bioplastic, polyhydroxyalkanoates (PHAs), polylactic acid (PLA) and other alternatives (Tabasi & Ajji, 2015). Bioplastics can be of both types of biodegradable and non-biodegradable as well. Biodegradable bioplastics are greatly diversified due to the difference present in biodegradation processes and rates. Just like Starch, cellulose, polyhydroxyalkanoates (PHAs), and polylactic acid (PLA) are some of these polymers (Sadaat et al., 2020).

PHAs (polyhydroxyalkanoates) are naturally and intracellularly produced in bacteria, these are biodegradable polyesters created by many bacteria as means of storing energy (the carbon and energy is stored in the form of granule) when the cells are limited in nutrients like phosphorus, nitrogen etc. The accumulation of PHA may be due to many

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external variables and internal limitations like restricted phosphorus, nitrogen or oxygen (Electron acceptors) availability. (Abd El-malek et al., 2020).

Polyhydroxyalkanoates (PHAs) are polyesters, known as the family of biodegradable and renewable polymers. They are regarded as green polymers of the future, along with polylactic acid, as both of them are anticipated to replace gradually the current plastics (Kourmentza & Kornaros, 2016).

PHAs can be of short chain lengths (having a chain length ranging 3–5 carbon atoms) such as P 4HB). The PHAs of medium chain length have 6-14 carbon atoms present for example, we have poly (3-hydroxyoctanoate) and Poly (3-hydroxyhexanoate). (Chee et al., 2010). Two of the most significant properties of the PHAs (polyoxoesters) are its thermoplasticity or its elasticity, containing R-hydroxyalkanoic acid (HA) monomers which are biosynthesized as carbon storage molecules and intracellular energy by the broad spectrum of Gram-negative and Gram-positive bacteria. PHA acts to be an excellent energy and carbon storage molecule, this is because of its insolubility in bacterial cytoplasm, causing just a little rise in osmotic pressure (Kourmentza et al., 2017).

Different PHA monomers are produced by various carbon sources because they are metabolized by many different metabolic pathways. By employing different waste streams as carbon sources, we should concentrate on lowering manufacturing costs. Among these are whey, starch, used coffee grounds, wastewater, rice straw, glycerol and lignin (Phithakrotchanakoon et al., 2015).

The most cost-effective and one of the most common carbon sources used is molasses which is an industrial waste material produced in bulk, it can be made from sugarcane or beet. The composition of molasses varies depending on the source from where it is derived such as cane molasses consists of 31-40% of sucrose & beet molasses consists of 60-63% sucrose. The polysaccharides content was found to be 1.5-4% in cane molasses and 1-2% in beet molasses. Thus, showing high carbohydrate content, can be used as a cheap carbon source alternative for PHA production (Kumar et al., 2019).

Proteus mirabilis is a gram-negative bacterium that has been identified as PHA producer in many studies. The screening of bacterial strains revealed the production of PHA in Proteus mirabilis but along with other strains such as Bacillus sp., Pseudomonas sp. The PHA production was found to be in lower to moderate quantities from Proteus mirabilis in previous studies (Patel et al., 2012). The data available for production & extraction of PHA is less in terms of Proteus mirabilis. In this conducted study, the strain E5 was found to be Proteus mirabilis and was a positive bacterial isolate for PHA production. Therefore, Proteus mirabilis was selected in this study to assess the yield of PHA as a highly promising bacterium for PHA production using molasses as a carbon source.

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2. MATERIALS AND METHODS

2.1 Collection of Bacterial Samples

The oil contaminated soil samples for bacterial isolation were collected from Hamid Auto workshop, Johar Town Lahore & Saeed Auto workshop, Township Lahore, Punjab, Pakistan.

2.2 Isolation of Bacteria

The soil sample 1g was added in the first test tube and further dilutions were prepared up to 10-9. The diluted samples of test tube with concentrations 10-3 10-4 10-5 were selected to isolate the bacterial colonies.

2.3 Spreading of Bacteria

The spreading of diluted bacterial samples was done evenly over the agar medium by using a sterile spreader. The plates were incubated at temperature 37°C for 24-48 hours to allow the bacterial growth.

2.4 Streaking of Bacterial Species

The samples were streaked using a hot loop, allowed to grow in an incubator for 24-48hrs. The resulting colonies were used for the microbial culture to grow on new plates and the fresh cultures were utilized for gram staining & further procedures.

2.5 Gram Staining Procedure

The colonies were picked to make a smear on the glass slides. The slides were heat-fixed and air-dried. The staining reagents used were crystal violet & Gram's iodine. The slides were flooded with decolorizing agent. The counterstain safranin was added, the washing of slide was done until there was no color. The absorbent paper was used to blot dry, and the results were observed using a Brightfield microscope. The oil immersion was used to get better results. Gram negative bacteria showed the pink or red stain whereas the gram-positive bacteria gave the results of blue or purple stains.

2.6 Nile Blue Staining

Nile blue staining technique was used to screen bacteria which have the potential to produce PHA. The bacteria were grown on the PHA producing medium only. The bacteria producing PHA will only show the fluorescence under UV transilluminator. For the preparation of media, macronutrients & micronutrients were added along with glucose as a carbon source. The Nile blue solution was made by adding Nile blue powder 0.5 mg in 1ml methanol. In media, 75µl of Nile blue was added (Mascarenhas & Aruna, 2017).

The glucose solution was made freshly by adding 3g glucose in distilled water (10ml). Then, glucose was added as a carbon source through syringe filtration to avoid crystallization.

At the end 2.25g agar was added to solidify the media. The media was poured in the plates & was allowed to solidify for 20 minutes. The fresh culture colonies were used to

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be streaked on the plates after labelling them. The plates were incubated at 37°C for 24-48 hrs (Mascarenhas & Aruna, 2017). The bacterial growth was observed the next day & out of 10 bacterial strains 2 showed fluorescence under UV light in the lab. It was confirmed that 2 of the strains were able to produce PHA.

2.7 Sudan Black B Staining Method for Screening of Bacteria

Sudan Black B method of staining was applied to identify the bacteria having the potential to produce the poly- hydroxyalkanoates. Initially, in this procedure we utilized SBB solution (3%) in 97% ethanol. A microbial broth was prepared and was fixed on a glass slide. Then sudan black B solution was stained for 10 minutes. The SBB solution was prepared by adding 3mg sudan black powder in 70% ethanol. Xylene was used to immerse the slide until it was decolonized completely. Safranine was used as a counterstain for 10 seconds, then washed & dried to examine the cells in optical microscope. The visual screening by Sudan Black B gave us positive PHA producers and negative accumulators of PHA. The positive was stained dark blue or black whereas the negative ones remained light blue or white under the microscope (Aragosa et al., 2021).

2.8 Selection of Positive Bacterial Isolates

The bacterial strains which were able to produce PHA & produced the highest amount of PHA were selected for further analysis. Out of 10 bacterial strains 2 were found to give positive results. These samples were stored at 4ºC so that subculturing can be done every 15 days. These samples were further used for the production & extraction of PHA.

2.9 Identification & Analysis of Bacterial Strains

The next step was to identify the bacterial isolates showing positive results for PHA production. The API strips were used to identify and confirm the bacterial species which were able to produce PHA from this oil contaminated soil samples.

2.10 Production of Polyhydroxyalkanoates

The preparation of standard inoculum included the inoculation of nutrient broth 150ml in a conical flask (250 mL). The incubation of these inoculated flasks was performed on a rotatory shaker at 150 rpm with temperature ranging 35-37ºC for 24 hours, this inoculum was used for further experiment. The cells were harvested after 24 hours of cultivation by centrifugation (10,000 rpm) at temperature 4ºC for 15 min. The sterile distilled water was used for washing aseptically and resuspended (Chaudhry et al., 2011).

2.11 Time Profiling

The Spectrophotometer was used to measure the OD values to check the bacterial growth after regular intervals.

2.12 Preparation of Media for Bacterial Growth

The macronutrients & micronutrients were added in all the flasks required in the experiment. The quantities of micronutrients & macronutrients were according to g/L. The

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distilled water was added along with the nutrients after weighing them. As we prepared 150 ml media in each flask, the quantities added in the media were as shown in Table 2.1.

Table 2.1: Quantities of micronutrients & macronutrients required for media preparation

Macronutrients g//L		**Micronutrients g/L**	
NH_4cl	0.15	$ZnSO_4$	0.012
$NaHCO_3$	0.075	$Mncl_2$	0.0045
KH_2PO_4	0.3	H_3BO_4	0.045
Na_2HPO_4	0.3	$Cocl_2$	0.03
$MgSO_4$	0.075	$Cucl_2$	0.0015
$Cacl_2$	0.0015	$Nicl_2$	0.003
Citric acid	0..0075	Na_2MoO_4	0.0045

2.13 Addition of Strain

A nutrient broth of strains was prepared by adding 0.4g nutrient broth in 50ml distilled water. The broth was autoclaved & the fresh culture colonies were inoculated in the broth. The strains in the broth were allowed to incubate overnight. The process of incubation was carried out in Erlenmeyer flasks 250 ml and production medium was 150 ml each. The incubation occurred at rotatory shaker 150 rpm. There was no discard of solution PHA production starts.

The centrifugation of the solution after measuring the OD values was done at 10,000 rpm for 15 minutes at 4°C. The pellets were formed & stored at 4°C for extraction of PHA. These pellets were weighed after drying. The optical density was measured by spectrophotometer at 600 nm to monitor the cell growth or analyze the growth effect. The standard calibration curve showed the bacterial growth & production of PHA. The bacteria showed significant growth with the increase of incubation time. The contents were centrifuged, washed and a pellet was obtained to further extract and calculate the yield of PHA.

2.14 Glucose as a Carbon Source

The glucose was added in flasks to be utilized as carbon source to analyze the bacterial growth. The glucose solution was prepared freshly to be added through syringe filtration.

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2.15 Estimation of Molasses as a Carbon Source

In this experiment, sugar cane molasses obtained from the sugar factory Nawab fields near Lahore, Pakistan was used as a carbon source with concentration of 2%. The carbon source used was one of the major factors accumulating PHA production. The total carbohydrate content should be known in the carbon source used for PHA production therefore, estimation of molasses was necessary to get the carbohydrate content present in it. Phenol sulfuric acid method was used as a rapid colorimetric process for determination of total sugar or carbohydrate content present in a sample, the sulfuric acid has the ability to break down all disaccharides, polysaccharides or oligosaccharides into the simple sugars (monosaccharides). In this experiment, the known concentrations such as 0.2, 0.4, 0.6, 0.8, 1 mg/ml were used to determine the carbohydrate content by taking the OD values & generating a graph.

2.16 Extraction of PHA

The pellets stored after bacterial growth were utilized for the extraction of PHA. The pellets were stored at 4°C & after being dried its dry cell weight was measured.

SDS was used for extraction of PHA. In our experiment, we made SDS solution by adding 5g in 95 ml distilled water. The SDS solution was added in the tubes containing pellets. The amount of SDS to be added was determined according to the DCW.

The resuspension was done after adding SDS solution & was placed for incubation (1 hour) at room temperature. After incubation, tubes were placed in the water bath at 95°C for 1 hour so that the cellular components break down completely.

The tubes were centrifuged at 13000xg (9000rpm) for 10 minutes. The pellets were formed and washed with water. The tubes were centrifuged again with same conditions 4-5 times to eliminate the excess foam produced due to addition of SDS. The pellets were resuspended again.

The pellet suspension was transferred to glass tubes & chloroform was added 3ml in each glass tube. After 20 min, a layer is formed which contains PHA. The chloroform layer content is transferred to a plate and is kept there for drying for 24 hours (Chaudhry et al., 2011).

The dried PHA is then measured to calculate the actual yield of PHA. After drying, the calculation of the yield of PHA was done by using the formula as under:

Percentage of PHA = Weight of PHA / Dry cell weight × 100.

Percentage of PHA using *glucose* as carbon source = $\frac{\text{weight of PHA}}{\text{dry cell weight}}$ ×100.

$$= \frac{0.0024}{0.172} \text{ x } 100$$

$$= 13.023\ \%$$

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Percentage of PHA using *molasses* as carbon source = $\frac{\text{weight of PHA}}{\text{dry cell weight}}$ ×100.

$= \frac{0.013}{0.051}$ x 100

= 25.49 %

2.17 FTIR Analysis

FTIR (Fourier Transform Infrared spectroscopy) was the technique to identify the chemical structure of the polyhydroxyalkanoates that were extracted in the extraction process from the bacterial sample. The solvent was evaporated by dissolving in chloroform. FTIR spectrophotometer gave the infrared spectra (wave number range 650 to 4000 cm-1) of the samples to determine the structure and bonds between them & to predict their structure.

3. RESULTS

3.1 Isolation of Bacteria from Soil Samples

The bacterial samples collected were diluted and streaked on the agar plates to get the bacterial growth as shown in figure 3.1. The colonies formed after incubation were picked for the gram staining to identify which strains were gram negative & which were gram positive.

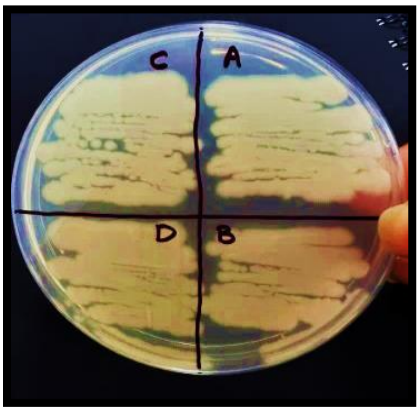

Figure 3.1: Formation of bacterial colonies isolated from soil samples after streaking on agar plate with 24-48 hours of incubation at 37ºC

3.2 Gram Staining of Bacterial Strains

The 10 bacterial strains labelled as A1, B2, C3, D4, E5, F10, G12, H55, I14, J16 were observed under microscope on 100X using oil immersion to increase the resolving power of the image. Out of 10 strains, only 2 were found to be gram positive whereas all others were found to be gram negative (showing red or pink stains). The strains B2 & C3 were observed gram-positive rod-shaped bacteria. The strains A1, D4, E5, F10, G12, H55, I14 & J16 were rod-shaped gram-negative. Figure 3.2 depicts the microscopic view of 4

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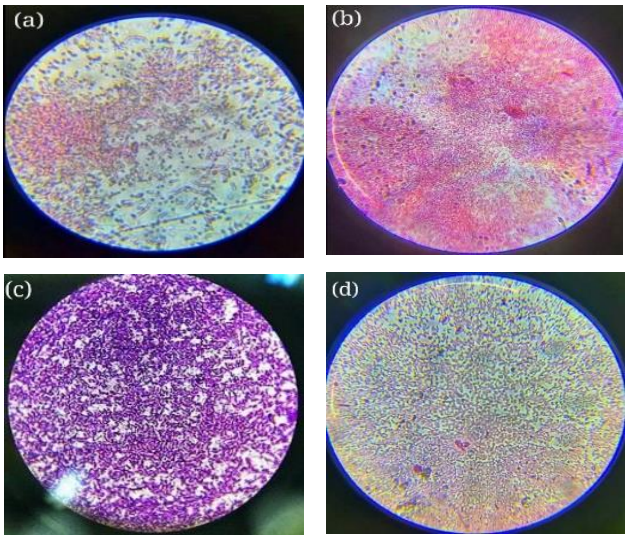
bacterial strains in which only C3 was observed as gram positive bacteria whereas other were gram negative.

Figure 3.2: Gram staining of bacterial isolates seen under microscope with 100x magnification to identify Gram-positive & Gram-negative bacteria. (a) Gram negative bacterial isolate A1 (b) Gram negative bacterial isolate D4 (c) Gram positive bacterial isolate C3 (d) Gram negative bacterial isolate E5



3.3 Screening of PHA Producers by Nile blue Staining

The figure 3.3 illustrates the results of Nile blue staining under UV light in which only two of the 10 strains showed the fluorescence when grown on PHA producing media. The strain C3 & strain E5 gave the fluorescence under UV transilluminator. The results revealed that strain C3 & strain E5 have the potential for the production of PHA. The other strains did not show any fluorescence when observed under UV light.

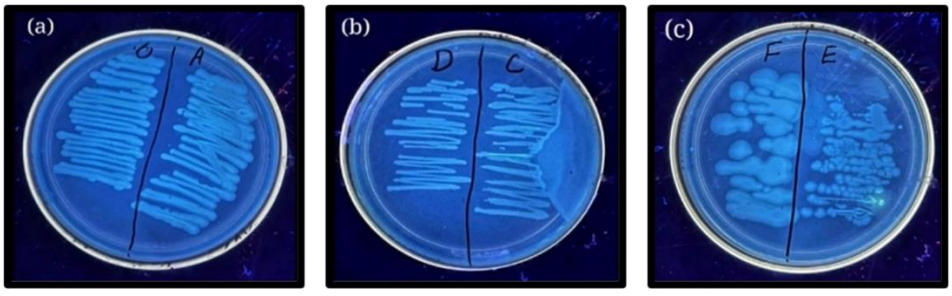


Figure 3.3: Nile Blue staining of bacterial isolates for screening of PHA producers & non-PHA producers observed under UV light (365 nm). (a) The strains A1 & B2 showed no fluorescence (b) The strain C3 showed fluorescence & strain D4 did not showed any fluorescence (c) Fluorescence detected in strain E4 & there was no fluorescence detected in isolate F10

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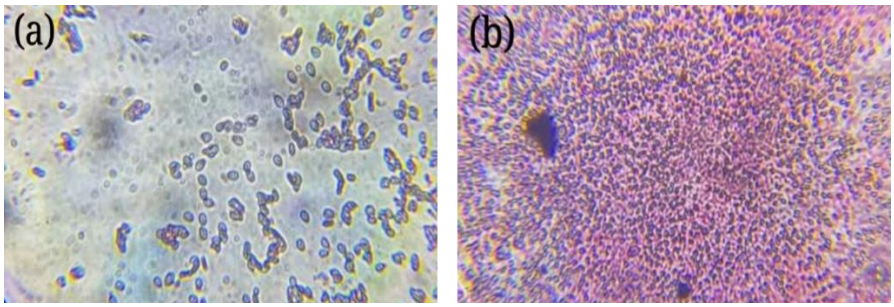
3.4 Sudan Black B Staining for Confirmation of Positive PHA Producers

Sudan Black B is a fat-soluble dye, and its staining results confirmed the potential positive PHA accumulators and the negative accumulators. The positive producers were stained black or dark blue whereas the other remained white having no potential to produce PHA. Sudan black B results gave the confirmation that strain C3 & strain E5 as shown in figure 3.4 are able to accumulate PHA. Later, API strips identified these two strains to be Bacillus sp. & Proteus mirabilis.

Figure 3.4: Microscopic Analysis of Sudan Black B Staining at 100X magnification for the confirmation of PHA accumulating bacterial strains. (a) PHA granules synthesized in strain C3 (b) PHA granules synthesized in strain E



3.5 Phenol-Sulphuric Acid Test for Carbohydrate Estimation

Molasses was used as a carbon source in this study therefore, carbohydrate content present in it was estimated using phenol-sulphuric acid test. Figure 3.5 depicts the known glucose concentrations such as 0.2, 0.4, 0.6, 0.8, 1 mg/ml and a trend line of graph was generated against the OD values. This trend line helped to measure the carbohydrate concentration in the substrate such as molasses. The value of 29.4 mg/ml was predicted from this standard curve of glucose.

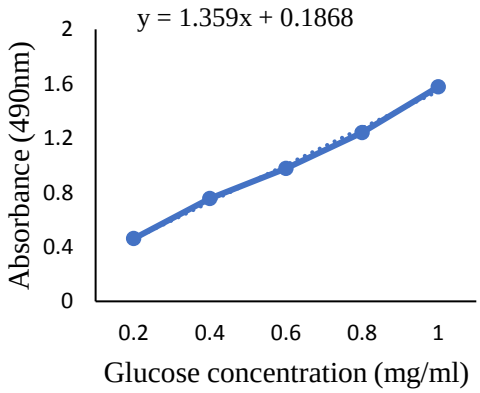


Figure 3.5: Estimation of carbohydrate content (glucose) in molasses utilized as a carbon source in this study of PHA production from Proteus mirabilis

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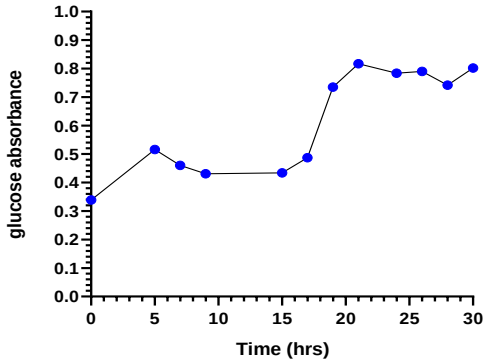
Figure 3.6: Time profiling of Proteus mirabilis enriched with glucose as a carbon source



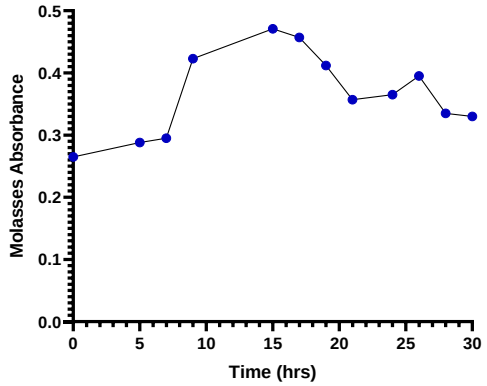
3.6 Time Profiling of *Proteus Mirabilis* Utilizing Glucose as a Carbon Source

Proteus mirabilis was allowed to grow using glucose as a carbon source. Figure 3.6 illustrates the growth curve of Proteus mirabilis of 30 hours. The OD was 0.339 at 0 hours and showed an increase after 5 hours (0.516). The growth curve was maximum 0.817 after 21 hours, 0.784 after 24 hours and 0.802 after 30 hours. Hence, the bacterial growth increased with increased incubation time till 30 hours when glucose was used as carbon source for Proteus mirabilis.

3.7 Time Profiling of *Proteus mirabilis* Utilizing Molasses as a Carbon Source

Molasses was used as a carbon source in crude form without any pre-treatment. Figure 3.7 shows the growth of *Proteus mirabilis* utilizing molasses with respect to time (30 hours). The OD at 0 hours was 0.265 and was found to be maximum at 15 hours & 17 hours which is 0.471 & 0.457. The growth of *Proteus mirabilis* was found to be decreased after 21 hours of incubation.

Figure 3.7: Growth pattern analysis of Proteus mirabilis enriched with molasses as a carbon source



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3.8 Extraction of Polyhydroxyalkanoates from Proteus Mirabilis

The pellets obtained by SDS digestion method were dried and used for the extraction of PHA. The PHA content in plate as shown in figure 3.8 was measured to calculate the percentage of PHA extracted from Proteus mirabilis utilizing glucose & molasses as carbon sources. Proteus mirabilis was able to produce low to moderate quantities of PHA. The PHA content obtained using molasses as a carbon source (25.49 %) was higher as compared to glucose (13.023 %).

3.9 Comparative Analysis of DCW, PHA Content & Residual Biomass

Figure 3.9 shows the comparison between the PHA content obtained, dry cell weight, residual biomass & percentage of PHA. The values differ utilizing two different carbon sources such as glucose & molasses in this study. Proteus mirabilis gave dry cell weight 0.566 g/L & PHA content 0.074 g/L while using glucose. However, the % of PHA obtained was found to be 13.02%. By using molasses as a carbon source, DCW was found to be 0.166 g/L, PHA content 0.043 g/L and PHA % was 25.49%. Hence, the PHA obtained was more using molasses (25.49%) as compared to glucose (13.02%), giving the results that Proteus mirabilis was able to produce minute to moderate quantities of PHA in this study.

3.10 T-test Applied on Bacterial Growth Patterns of Proteus Mirabilis

The statistical t-value was obtained after comparing the bacterial growth. The associations and variations were analyzed by applying t test. $P < 0.05$ was considered statistically significant. The P value was found to be 0.0002 which was statistically significant ($P < 0.05$). The OD values showed a difference at the initial and final time of incubation as shown in figure 3.10. The values of bacterial growth at 9, 15, 17 hours were found to be constant such as 0.423 and 0.431.

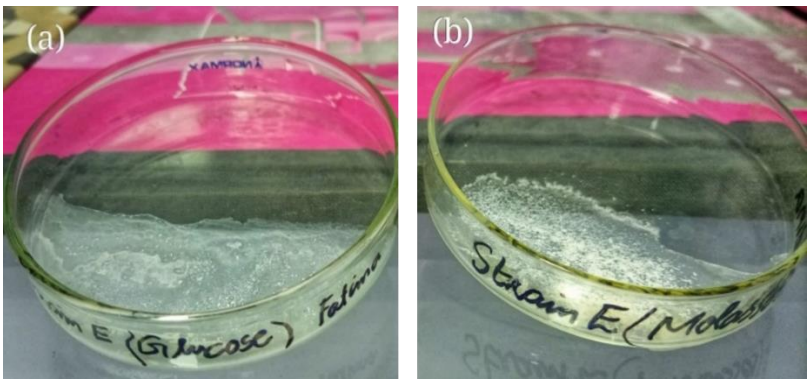

Figure 3.8: Extracted PHA from dried pellets obtained by SDS digestion & chloroform extraction method. (a) PHA extracted from Proteus mirabilis using glucose as a carbon source (b) PHA extracted from Proteus mirabilis using molasses as a carbo source

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Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 04 | 2023
DOI 10.17605/OSF.IO/6AMUQ

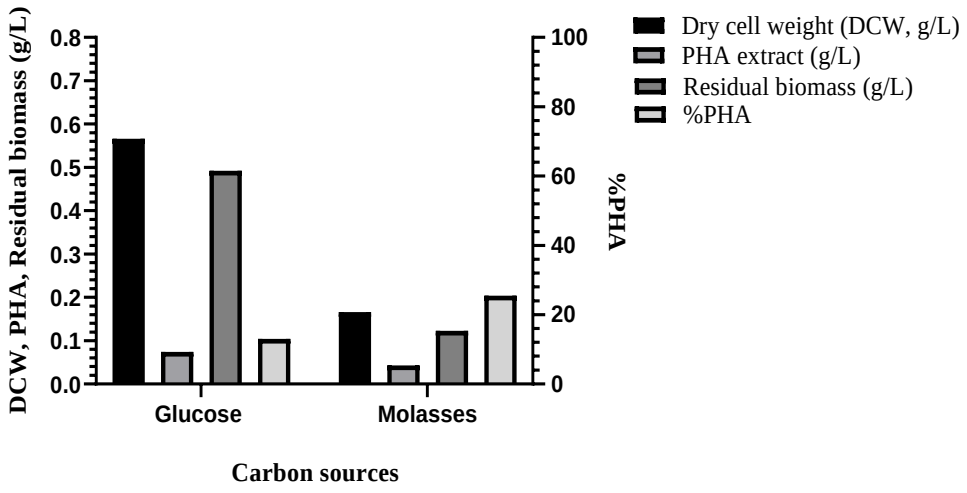
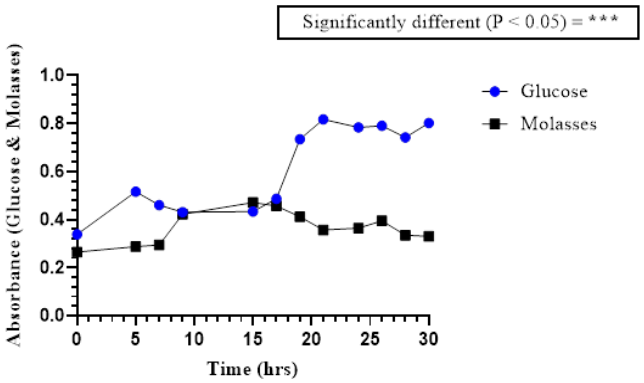


Figure 3.9: Comparison of dry cell weight, PHA contents, residual biomass (g/L) & % PHA of Proteus mirabilis using Glucose & Molasses as carbon sources after incubation time of 30 hours

Figure 3.10: Analysis of significant difference of growth curves of Proteus mirabilis with respect to time interval (30 hours)



3.11 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Fourier transform infrared spectroscopy (FTIR) is a chemical analysis used to determine the molecular structure of the chemical compound present in the sample. The IR spectrum contributes to distinguishing the chemical components of the sample & its concentration. FTIR spectroscopy is frequently used because of its several advantages such as minute quantities of samples are required, analysis time is less (30minutes) & sample manipulation is minimal.

FTIR spectroscopy was performed in the present study between the frequency ranges of 4000- 650 cm−1 to analyze the molecular structure & functional groups. IR spectra revealed the intense absorption at wave numbers 1637 cm−1 and 1647 cm−1 indicating

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the presence of ester carbonyl (C=O) stretching groups as compared with the standard polyhydroxyalkanoates (figure 3.11). The marked peaks at wave numbers between 1600-1300 cm−1 (1541 cm−1) showed the presence of O-H groups. The adsorption bands recorded at 687 cm−1, 697 cm−1 and 709 cm−1 revealed the presence of C-H groups. The band depicting the adsorption at 1226 cm−1 represents the -CH3 group. Other absorption bands such as at 2350 cm−1 and 2376 cm−1 can be associated with sugar derivatives and the β-glycosidic linkages between the monomers of sugar.

4. DISCUSSION

Environmental issues are affecting the entire world nowadays therefore, it is the need of the hour to deal with solid waste management and global warming by using natural alternatives such as use of polyhydroxyalkanoates to make biodegradable plastics. Several bacteria have been used to produce PHA under stress conditions using cheap carbon substrate.

Proteus mirabilis is a bacterial strain having potential to produce PHA even under stress condition. In this study, the PHA content obtained is 13.02% using glucose as a carbon source having dry cell weight 0.56 g/L. Whereas in previous study in Thailand, five bacterial isolates were identified Pseudomonas, Bacillus, Proteus, Aeromonas & Cupriavidus as potential PHA producers. Proteus mirabilis produced DCW 1.87 g/L & PHA production was found to be non-detectable. The difference in DCW & less PHA production might be due to change in the samples or different incubation time. In this study, samples from oil contaminated sites were used and in the previous one wastewater sample was used for the isolation of bacteria (Sangkharak & Prasertsan, 2012).

The isolation & screening of PHA producing bacteria was carried out in this study from soil samples and out of 10 bacterial strains 2 strains showed fluorescence in Nile Blue staining and was found to belonging to Bacillus sp. & Proteus sp. Proteus mirabilis was gram negative rod-shaped bacteria. In the past study in Malaysia, the samples of soil sediments near hotsprings were used for isolation of potential PHA accumulators. The morphological & biochemical analysis showed affiliation of bacterial isolates to Salmonella sp., Proteus sp., Shigella sp., Enterobacter sp., and Klebsiella sp. Hence, the PHA content from Proteus mirabilis was also found to be low in quantity as in this study (Lee & Rahim, 2020).

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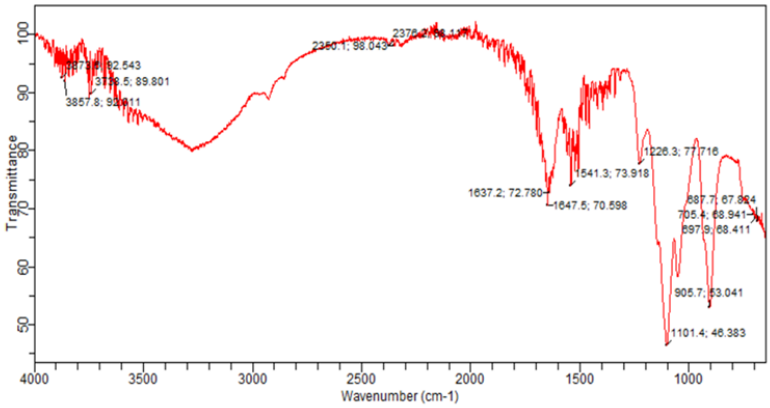

Figure 3.11: Fourier transform infrared spectra (FTIR) analysis of PHA granules produced by Proteus mirabilis

Proteus mirabilis strain was able to utilize fructose & glucose as a carbon source, PHA production was found to be varying from moderate quantities 0.345 g/l to lower quantities 0.015 g/l in previous research conducted in India. In this study, Proteus mirabilis used glucose & molasses as a carbon source, hence also produced low quantities of PHA 0.074g/l from glucose & 0.043g/l from molasses. Moreover, the previous studies revealed that proteus has potential to produce PHA in moderate to low quantities (Porwal et al., 2008).

A study conducted in Turkey gave the maximum yield of 49.12% using cane molasses. The strains of pseudomonas, bacillus & proteus were identified from the mixed microbial cultures and were tested positive for presence of PHB granules by Sudan Black staining. The maximium yield was obtained by supplementing 3% corn steep liquor whereas in this study the maximum yield using cane molasses was 25.49% after identifying proteus mirabilis strain as PHA producer by Sudan black staining. The difference in the yield might be due to the use of corn steep liquor as a substrate in the previous study. Therefore, the use of CSL enhanced the production of PHA (Ceyhan & Ozdemir, 2011).

The two strains identified in this study were Bacillus sp. and Proteus mirabilis by API strips. Proteus mirabilis was further selected to check the production of PHA after confirmation with Sudan Black staining. The PHA content was 13.02% using glucose & 25.49% using molasses whereas in Italy the study was conducted earlier to isolate the PHA producing bacteria from soil samples of southern region. The Methylene Blue method was used for identification of bacteria, then biochemical analysis & susceptibility tests identified the strains belonging to genus Proteus and Serratia, giving the final yield of PHA as 64.7%. The reason for the increased yield might be due to the mixed cultures of genus Proteus with Bacillus and Enterobacter in the previous study (Aragosa et al., 2021).

This current study focused to draw attention towards use of bioplastics as an alternative for traditional plastics & to screen bacterial strains accumulating maximal amount of PHA

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E-Publication: Online Open Access
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at limiting conditions. This study also assessed the feasibility of bacterial strains capable of producing PHA. Thus, Proteus mirabilis isolated from oil contaminated soil samples was able to produce PHA in low to moderate quantities and can be used as an alternative to produce bioplastics.

5. CONCLUSION

The novel bacterial strain Proteus mirabilis was isolated from the oil contaminated sites in this study for PHA production using molasses as a carbon source, giving moderate accumulation of PHA. The bacterial isolate was identified and confirmed as a positive PHA producer. The PHA accumulated was cost effective and can easily be used as a substitute for petrochemical derived plastics. Moreover, more work is needed for the economic assessment & to evaluate the feasibility of this strain for large-scale manufacturing of PHA.

References

1) F. Abd El-malek, H. Khairy, A. Farag, and S. Omar, “The sustainability of microbial bioplastics, production and applications”, *Int. J. Biol. Macromol*, vol. 157, pp. 319–328, 2020.

2) C.R. Alvarez-Chavez, S. Edwards, R. Moure-Eraso, and K. Geiser, “Sustainability of bio-based plastics: General comparative analysis and recommendations for improvement”, *J. Clean. Prod*, vol. 23, no. 1, pp. 47–56, 2012.

3) A. Aragosa, V. Specchia, and M. Frigione, “Isolation of two bacterial species from argan soil in Morocco associated with polyhydroxybutyrate (PHB) accumulation: Current potential and future prospects for the bio-based polymer production”, *Polymers*, vol. 13, no. 11, 2021, Art. no. e1870.

4) E.B. Arikan, and H.D. Ozsoy, “A review: Investigation of bioplastics”, *J. Civ. Eng. Archit*, vol. 9, no. 1, pp. 188–192, 2015.

5) N. Ceyhan and G. Ozdemir, “Poly-β-hydroxybutyrate (PHB) production from domestic wastewater using Enterobacter aerogenes 12Bi strain”, *Afr. J. Microbiol. Res*, vol. 5, no. 6, pp. 690–702, 2011.

6) W.N. Chaudhry, N. Jamil, I. Ali, M.H Ayaz, and S. Hasnain, “Screening for polyhydroxyalkanoate (PHA)-producing bacterial strains and comparison of PHA production from various inexpensive carbon sources”, *Ann. Microbiol*, vol. 61, no. 3, pp. 623–629, 2011.

7) J.Y. Chee, Y. Tan, M.R. Samian, and K. Sudesh, “Isolation and characterization of a Burkholderia sp. USM (JCM15050) capable of producing polyhydroxyalkanoate (PHA) from triglycerides, fatty acids and glycerols”, *J. Polym. Environ*, vol. 18, no. 4, pp. 584–592, 2010.

8) E. Karana, “Characterization of ‘natural’and ‘high-quality’materials to improve perception of bio-plastics”, *J. Clean. Prod*, vol. 37, pp. 316–325, 2012.

9) C. Kourmentza, and M. Kornaros, “Biotransformation of volatile fatty acids to polyhydroxyalkanoates by employing mixed microbial consortia: The effect of pH and carbon source”, *Bioresour. Technol*, vol. 222, pp. 388–398, 2016.

10) C. Kourmentza, J. Plácido, N. Venetsaneas, A. Burniol-Figols, C. Varrone, H.N. Gavala, and M.A. Reis, “Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production”, *J. Bioeng*, vol. 4, no. 2, 2017, Art. No. 55.

ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 04 | 2023
DOI 10.17605/OSF.IO/6AMUQ

11) P. Kumar, A. Maharjan, H.B. Jun, and B.S. Kim, “Bioconversion of lignin and its derivatives into polyhydroxyalkanoates: Challenges and opportunities”, *Biotechnol. Appl. Biochem*, vol. 66, no. 2, pp. 153–162, 2019.

12) N.S. Lee, and A.A. Rahim, “Isolation and Characterization of Poly-hydroxy alkanoates (PHAs) Producers from Kg Batu Melintang hotspring”, *J. Trop. Res. Sustain. Sci*, vol. 8, no. 1, pp. 1–4, 2020.

13) J. Mascarenhas, and K. Aruna, “Screening of poly-hydroxy alkonates (PHA) accumulating bacteria from diverse habitats”, *J. Global.Biosci*, vol. 6, no. 3, pp. 4835–4848, 2017.

14) S.K. Patel, M. Singh, P. Kumar, H.J. Purohit, and V.C. Kalia, “Exploitation of defined bacterial cultures for production of hydrogen and polyhydroxy butyrate from pea-shells”, *Biomass. Bioenergy*, vol. 36, pp. 218–225, 2012.

15) N. Peelman, P. Ragaert, B. De Meulenaer, D. Adons, R. Peeters, L. Cardon, F.V. Impe, and F. Devlieghere F, “Application of bioplastics for food packaging”, *Trends. Food. Sci. Technol*, vol. 32, no. 2, pp. 128–141, 2013.

16) C. Phithakrotchanakoon, V. Champreda, S. Aiba, K. Pootanakit, and S. Tanapongpipat, “Production of polyhydroxyalkanoates from crude glycerol using recombinant Escherichia coli”. *J. Polym. Environ*, vol. 23, no. 1, pp. 38–44, 2015.

17) S. Porwal, T. Kumar, S. Lal, A. Rani, S. Kumar, S. Cheema, H.J. Purohit, R. Sharma, S.K.S Patel, and V.C. Kalia, “Hydrogen and polyhydroxybutyrate producing abilities of microbes from diverse habitats by dark fermentative process”, *Bioresourc. Technol*, vol. 99, no. 13, pp. 5444–5451, 2008.

18) R. Sadaat, and N. Jamil, “Optimization of bioplastic production by Exiguobacterium sp. and Klebsiella sp. Using molasses as carbon source”, *J. Agric. Food*, vol. 1, no. 1, pp. 14–20, 2020.

19) K. Sangkharak, and P. Prasertsan, “Screening and identification of polyhydroxyalkanoates producing bacteria and biochemical characterization of their possible application”, *J. Gen. Appl. Microbiol*, vol. 58, no. 3, pp. 173–182, 2012.

20) R. Scaffaro, L. Botta, A. Maio, M.C. Mistretta, and F.P. La Mantia, “Effect of graphene nanoplatelets on the physical and antimicrobial properties of biopolymer-based nanocomposites”, Materials, vol. 9, no. 5, 2016, Art. No. 351.

21) R.Y. Tabasi, and A. Ajji, “Selective degradation of biodegradable blends in simulated laboratory composting”, *Polym. Degrad. Stab,* vol. 120, pp. 435–442, 2015.