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Research Article

Isolation and Screening of Polyhydroxyalkanoates (PHA) Producing Bacteria Utilizing Agricultural Waste

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Abstract

Polyhydroxyalkanoates (PHAs) are biosynthetic, environmentally friendly and biodegradable polyester stored as a granules inside the cytoplasm of microorganisms, granules are compounds of PHAs used as carbon and energy source, Synthetic polymer take many years to demolish completely, microorganisms can degrade PHAs within a year into carbon dioxide, water and energy, The main contributor for PHAs production cost is carbon sources cost, Accordingly it is favorable to produce PHA from any agriculture waste like rice bran. Aim of this dissertation is to utilize rice bran which was obtained from Limda field near Parul University, screening and isolation of polyhydroxyalkanoates PHAs producing bacteria, Synthesis of most effective PHB, different wild type microorganisms were studied by flask shaking method to determine their ability to produce PHA utilizing rice bran as carbon source, Total 16 isolates showed the fluorescence in the presence of Nile blue in solid medium under UV light, two bacterial isolates SF-3 and SF-2 isolated from jaggery waste, respectively, PHA Accumulation for (2%RB-1%) and (2%RB-5%) was 68% and 47% PHA/(CDW) respectively, the PHB obtain from (2%RB-1%) and (2%RB-5%) was analyzed by FTIR and NMR as poly hydroxyl butyrate (PHB).

Keywords: Polyhydroxyalkanoates; Polyhydroxybutyrate; Rice bran; Jaggery waste; Nile blue

Introduction

According to the last and most undertaking scenario that plastic waste management invented most fascinating scenario for production of biodegradable plastic, many biodegradable polymers that are similar to the petroleum plastic known already by researchers, which are available in the markets including polyethylene (PE), poly (p-phenylene) (PPP), polyhydroxyalkanoates (PHA), poly butylene succinate (PBS), poly (trimethylene-terephthalate) (PTT), poly(lactic acid) (PLA), application of biodegradable polymer due to their certain chemical and mechanical properties allows to be used in a great range of application, although, only polyhydroxyalkanoates can have valuable structural variation along with being monomer made in-vivo unlike the other polymers being chemically synthesized by in-vitro polymerize (Verlinden *et al*., 2007).

According to this study (PHAs) has been stored in the cytoplasm in the form of granules as well as others microorganism like Archea prokaryotic bacteria, Beijerinck was the first person to denote this structure in 1888 he used the microscope to observe the granules, (Braunegg *et al*., 1998), solely, chemical composition of PHA was determined in 1927 by the Lemoigne, he determined the capability of soil *bacterium, Bacillus megaterium* to breakdown the polymer under anaerobic condition into 3-hydroxybutyric acid (3HB) monomers (Keshavarz and Roy, 2010). So 3HB monomer was recognized as reserve molecule that created polymer like poly-3-hydroxybutyrate P (3HB) (Braunegg *et al*., 1998).

At the end of 1950, poly-3-hydroxybutyrate P (3HB) was viewed in other bacterial strain and in 1973 where P (3HB) was explained as being functionally similar to starch and glycogen (Sudesh *et al*., 2000), In 1974, There were the others polymers obtain along with P (3HB) with like poly-3-hydroxyvalerate P (3HV) withpoly-3-hydroxyhexanoate P (3HHx) to create copolymer. Different monomer can bind to make copolymer and many co monomer like co polyester etc. So copolymers have various chemical and mechanical properties such as crystallinity, melting point, glass transition temperature (Pederson *et al*., 2006). However, according to this study over 250 different bacteria producing biological polyester Polyhydroxyalkanoates (PHAs) containing gram positive and gram-negative species (Ojumu *et al*., 2004). However, PHAs are synthesized in the cytoplasm and store in the form of granules, and size of these granules are 0.2 – 0.7 mm, which is surrounded by a membrane layer containing lipid and protein about 2 nm thick. These contents can take up to 90% of the CDM (Cell Dry bio Mass) each inclusion has 1000 molecules (Braunegg *et al*., 1998; Yu *et al*., 2002).

addition, The PHA contents can be quickly conceived by the different instrumentation like to phase contrast light microscope as well as by staining with different dye like fluorescent oxazine, Sudan Black B, Nile blue A for identification (Sudesh *et al*., 2000). Granules full of PHAs serve as a store material for carbon and energy purpose, however, when microorganisms are under hunger condition such as lack of oxygen, less of nutrient, this oxygen and nutrient can protect the cell (Koller *et al*., 2010a).

PHA polymer are insoluble in water and also this molecule has optically active because it has hydroxyalkanoic acids (HA) R monomer configuration part, (Verlinden *et al*., 2007). And did not have any cis or Tran's configuration (Koller *et al*., 2010b). PHA have 200,000 to 3,000,000 Da molecular weight depends on to microorganism, it also depends to carbon amount and growing condition (Sudesh *et al*., 2000). Fig. 1, showed the general structure of PHA with other hydroxy alkanoates, monomers also have different variation, straight, side chain, saturated and unsaturated, side chain of some types are aliphatic or aromatic (Abraham *et al*., 2001). PHA polymer also have halo genic substituents that synthesized in vitro and vivo (Doi and Abe, 1990).

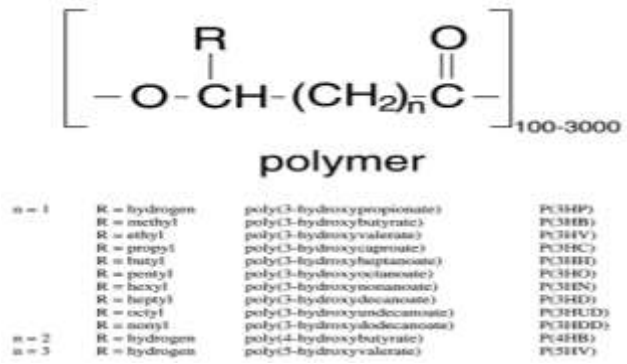

Fig. 1: Chemical structure of PHA synthesis by bacteria (Wu *et al*., 2003)

Polyhydroxyalkanoates have some great advantage over the synthetic plastics that microorganisms can break down it into small monomers to produce final products, PHAs were completely biodegradable into water and carbon dioxide, depend on to their biodegradable state, PHAs also do not like other petro chemicals or synthetic plastics to increase the landfill, PHAs can be demolished after use (Koller *et al*., 2010a).

Increasing of human population in the world, accumulation of non-degradable plastic waste among our planet, The accumulation of non-degradable waste has become great connection regarding the environments, synthetic plastic not only take many decades to degrade in environments, also produce toxins during demolishing, according to this purpose particular chemical compound should be used as a plastic which has similar properties to synthetic plastic (Devi and Nachiyar, 2011). Waste management and environmental protection agencies manufactured a great amount of chemical compound of polyhydroxyalkanoates that is biodegradable similar to conventional plastic, whole biodegradability without toxic waste and perfectly recyclable into organic waste (Gholami *et al*., 2016).

According to the application of biodegradability and properties of polyhydroxyalkanoates (PHAs) at various industrial and biomedical utilizing due to their biodegradability, resorb ability, compatibility, and piezoelectricity, however study showed that several bacteria species accumulating PHA compound, these bacteria can be isolated from different industrial waste material, *Pseudomonas genus* of bacteria are famous to accumulate PHA in presence of carbon source and suitable medium, so these bacteria is fed to fatty acid it passes through the beta oxidation biosynthetic pathway, to synthesize PHAs, therewith losing two carbon atom per each cycle, different published word use unsaturated fatty acid such as oleic acid to produce mcl-PHAs with a side chain of unsaturated monomer of fatty acid (Gumel *et al*., 2012).

According to annual estimation close to 150 million tones plastics are being consumed and the demand is getting increasing further more (Crank *et al*., 2004). Polyhydroxyalkanoates (PHAs are between biopolymer obtain by microbial fermentation, (Raj *et al*., 2014). However, study showed that PHA is polyester chemical compound and have the particular properties compared to petroleum derived polymer (Reddy *et al*., 2003). Physical properties including brittleness, glass transition temperature, melting point, molecular mass, PHA compound used in various places including photographic materials, bio plastic, drug delivery carriers, biofuels production, drug delivery carriers, antibiotics, It has been reported by varying the composition of carbon source, the physical and chemical properties of PHA can be change (Sav *et al*., 2014).

PHAs compound have similar properties to polypropylene and have the ability to reproduce in wide range in environment through bacteria, (Valappil *et al*., 2007). Colombia has huge variety of carbon-rich fruit plant which has the chemical compound that is use for bio plastic including Hymenaea courbaril and can be available in Spain, Portugal, Arabia, Somalia and West Indies (Aalzate *et al*., 2008).

Fique juice contains potassium (0.03%), proteins (0.96%), phosphorus (0.02%), 3% total sugars, proteins (0.96%), trace amounts of sodium, cooper, iron, zinc, etc. However, the purpose of this study to determine the production of PHA from these plants by the help of *Bacillus megaterium* from carob pulp and fique juice as the sole carbon source, sugar cane molasses, and Glucose were used as a reference and control, respectively, of inexpensive carbon substrate for production of PHA, its extensive use in the biopolymer industry is restricted by the food and biodiesel industries (Salazar *et al*., 2013). PHA molecule consist of 3-hydroxy fatty acids in the following figure.

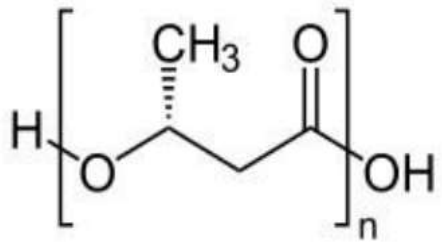

Fig. 2: Chemical structure of PHA

Various bacterial species such as also have the ability to produce PHA by the fermentation process including *Escherichia coli*, *Bacillus sp*, *Alcaligenes*, *latus*, and *Ralstonia eutropha*, etc (Wang and Lee, 1997). PHA can be obtained by the both prokaryotic and eukaryotic bacteria (Poirier, 2002). more than 75% content obtained by the fermentation process, The production cost of PHA is still high compare to synthetic polymer production, however, the good way to reduce the cost of production utilizing low cost waste material (raw material) contain industrial wastes, agriculture wastes, starchy wastewater, milk whey, agro-industrial oil, all this waste material is use as carbon source in fermentation process to produce PHA (Thomas S *et al*., 2013).

Materials and Methods

Collection of Raw Materials

Rice bran were collected from limda field, Vadodara, Gujarat, India. Isolated colonies were also obtained from jaggery waste, jaggery waste was collected from surat, Kamrej, Vadodara, Gujarat, India.

Isolation of Bacterial Strains

The jaggery waste sample was collected from various site of sugar industry, surat, kamrej, Gujarat, India, the amount of jaggery sample was around 1kg, 10 fold dilution of the jaggery waste sample were made and 0.1 ml of each was surface spread onto the nutrient agar, composition of nutrient agar medium used is as a follows pH=7.4±0.2), sodium chloride, 5.0 g; yeast extract; agar 1.5 g; peptone, 5.0 g; beef extract, 1.5 g; the plates were incubated at 37C for 2 days and 36 colonies were isolated & purified further, plates were covered with paraffin and refrigerated.

Screening for Bacteria Utilizing Rice Bran

In order to characterize the ability of various bacteria to utilize the rice bran as carbon source to determine the growth of colonies and synthesis of PHA, the isolated colonies of different bacteria from jaggery source were incubated on nutrient agar plates and growth after 48 hours at 37°C, different colonies were streaked on solid minimal salt medium (MSM) plates including (1, 2, 3, 4, 5, 10% (w/v) of jaggery waste, the composition of jaggery waste contain predominantly made up of sucrose ($C_{12}H_{22}O_{12}$), with trace of mineral salt, iron, and some fiber, MSM media composition (in 1 l of the medium, pH=7.0); KH_2PO_4, 2.65; $(NH_4)_2SO_4$, 0.5g; $MgSO_4{\cdot}7H_2O$, 0.4g; $Na_2HPO_4{\cdot}12H_2O$, 9.65g, and 2g agar and Micronutrient composition including $ZnSO_4{\cdot}7H_2O$, 0.29 g; $FeSO_4{\cdot}7H_2O$, 2.78g; $CuCl_2{\cdot}2H_2O$, 0.17g; $MnCl_2{\cdot}4H_2O$, 1.98g; $MnCl_2{\cdot}4H_2O$, 1.98 g, Various isolated bacterial colonies from sugar bran were spreaded onto the solid minimal salt medium MSM, agar plates also containing micronutrient and different concentration of rice bran sample, incubated at 37°C temperature for 2 days, after 48 hours.

Screening for PHA Determination

Isolates utilizing Rice bran sample were streaked on minimal salt medium (MSM) containing rice bran and solution of 0.5mg Nile red in DMSO to reach final volume of 0.5µg dye (ml medium)−1, positive isolates colonies were seen under UV light, PHAs positive isolates were seen under UV light as florescence colonies, PHA Positive colonies were grown onto various concentration of rice

bran, increase of white reflection under UV light showed strong florescence and less white reflection showed weak florescence.

Verification of PHA Synthesis

PHA positive colonies were grown in five same 100ml flasks containing 100ml MSM media supplemented with (1 to 5%) rice bran, after autoclaving 100µl of micro nutrient solution was added in each flask and dissolved perfectly, Isolated were incubated in MSM media containing different concentration of rice bran, after 96h, the bacterial cell were harvested, cell growth was monitored gravimetrically, the cell pellet obtain from a fix culture was lyophilized and showed in terms of cell dry weight (CDW) (Table 1).

The four basic steps of the Gram stain used for isolated bacteria

1. Applied a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture.
2. The addition of iodine solution, which binds to crystal violet and traps it in the cell.
3. Rapid decolonization with ethanol, methanol and acetone.
4. Counterstaining with safranin.

After 20–40 min at 37°C, when the solution including biomass and sodium hypochlorite turns white, it was considered that all the cellular remnants are being digested. The PHA granules were collected by centrifugation. Pellet obtained was washed with water, methanol and acetone consecutively for the removal of the residual impurities by centrifugation at 8000rpm for 20 min. The pellet was then dissolved in chloroform and PHA film was obtained which was further analyzed by FTIR and NMR.

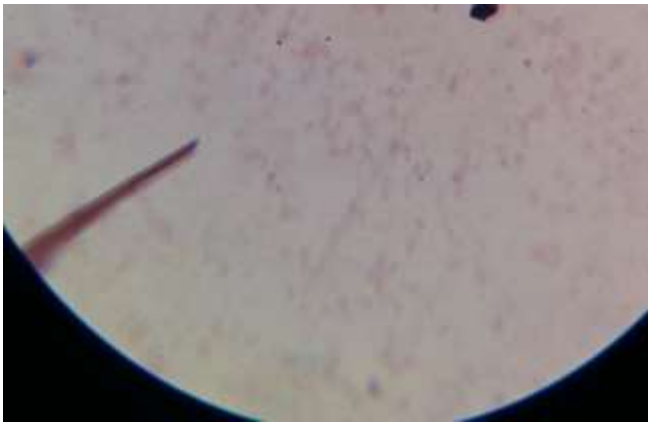

Fig. 3: Light microscope image of gram-negative rods (pink)

PHA Characterization

Fourier Transform Infrared Spectroscopy (FTIR). KBr pellet was used to obtain PHA production from (RB2, A7-1%, C22) to (RB2, A7-5%, A4), FTIR spectrometer used with spectral range between 4000 to 400 nm and recorded the IR spectra of PHA and other impurities, Nuclear Magnetic Resonance (NMR) 1H NMR spectra were acquired by mixing of polymer in deuterochloroform (CDCl3) at 10mg/ml concentration and analyzed by the usage of Bruker Avance II 500 spectrometer at 24C with 7.5ms pulse width (30◦ pulse angle), 1s pulse repetition, 10,340 Hz spectral width, 65,535 data points. Tetramethylsilane was used as an intrinsic shift standard.

Table 1: PHA synthesis by various bacterial isolates from jaggery

S.N.	Isolates	Cell dry weight (CDW) (mg)	PHA (mg)	PHA content expressed in % CDW
1	RB2, A7-1%, C22	215.6	146.6	68
2	RB2, A7-2%, A3	170	22.2	13.05
3	RB2, A7-3%, C24	160	40.8	25
4	RB2, A7-4%, A3	210.4	50.3	23.9
5	RB2, A7-5%, A4	200.2	94.1	47

Result and Discussion

Bacterial Isolates

Total 36 colonies were isolated from industrial sugar waste or jaggery onto the solid nutrient agar medium after overnight incubation at 37ºC temperature for further process.

Optimization of Rice Bran Concentration

Various bacterial isolates were spreaded on plates containing 5 different concentration of rice bran (1%, 2%, 3%, 4%, 5%), bacterial isolates utilizing rice bran showed high growth on all various five plates, chemical composition of rice bran was reported to containing (14% proteins, 15% fats , 8% fiber, 22% starch, 8% ashes well as a number of minor organic compound) (www.iralda.com) 20 different bacterial colonies were able to grow onto solid medium of rice bran (see Fig. 4), The bacteria which were able to utilize rice bran showed luxuriant growth onthe plates with 4% and 5% rice bran, but very less growth was observed on plates containing 1% and 2% rice bran.

Screening of the Isolates

Bacterial isolates which were able to grow on solid medium of rice bran containing different concentration were further screened for PHA synthesis, Nile red viable colony staining method was used to screen PHA producing bacterial isolates, Out of 20 jaggery isolates which were able to grow and utilize rice bran only 5 isolates showed fluorescence under UV light, the bacterial isolate which showed strong fluorescence were selected for PHA production and certain showed less positive fluorescence under UV light, finally the bacterial isolates from jaggery which were screened positive were, RB1, RB2, RB3, RB4, RB5 (Fig. 5).

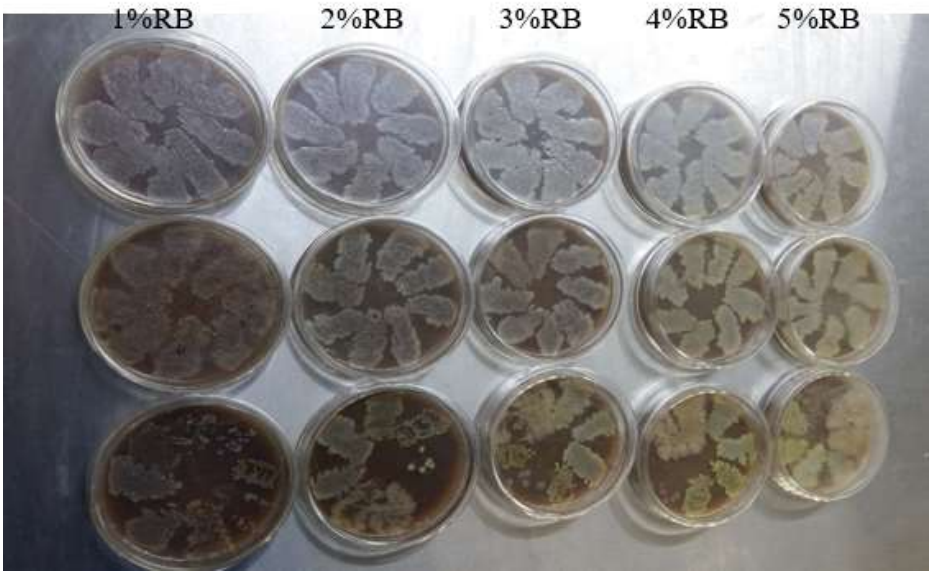

Fig. 4: Bacterial growth onto different concentration of rice bran after 48 hours

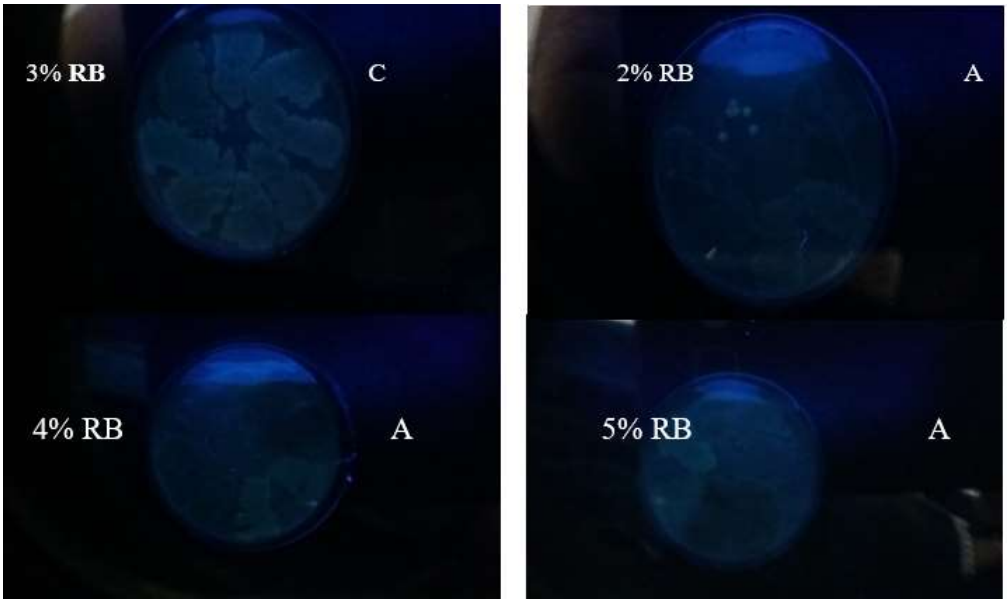

Fig. 5: Screening of bacterial isolates from jaggery waste source with Nile red viable colony staining method. The bacterial colonies showing fluorescenc Under UV light.

Confirmation of PHA Synthesis

16 bacterial isolates which were found promising while screening for PHA synthesis, showed strong fluorescence under UV light when grown on (2%RB) rice bran plates containing Nile Red dye, these isolates were further subjected for the confirmation of PHA production, isolate (RB2-1%) was found to be the most promising with PHA content of 68% cell dry weight (CDW) followed by isolate (RB2–5%) with 47% PHA/CWD, accumulation of PHA was almost comparable to which obtained from *Brevundimonas sp*. Strain reported by (Pammi *et al*., 2015). which accumulated PHA (61.86%) of CDW using rice bran, and also comparable to that obtained from *Bacillus subtilis* reported by (Anjali *et al*., 2013). that 58.59% (w/w) of (CDW) accumulated PHA obtained by the sugarcane molasses, while in present research we have obtain PHA accumulation up to 68% percent utilizing rice bran which contain other impurities along with PHA accumulation.

FTIR analysis of the biopolymer from (RB 1%) and (RB 5%) compared with the standard PHB (sigma) revealed absorption bands at 1724cm−1, comparable to the ester carbonyl group of PHA as shown in (Fig. 7) Characterization of PHA.

Fig. 7 shows 1H NMR and FTIR, NMR shows the characteristics 500 MHz1H NMR spectra for isolated of PHA from SF-3 compared with standard PHB from sigma which shows the above suitable resonance signals, -HC=CH at 5.30 ppm, -CH2-COOH at 2.50ppm, methylene groups ranging from 1.25 to 1.57ppm, and a terminal –CH3 at 0.9 ppm shows that the PHA produced is polyhydroxybutyrate, which was almost similar to standard PHB (sigma), based on the characterization of PHA produced by SF-3 through NMR, FTIR and its comparison with the standard PHB sigma, it was observed that the PHA obtain from RB2-1% is having the same properties to that of standard PHB sigma.

Fig. 6: Polyhydroxybutyrate (PHB) film obtained by RB2 isolate

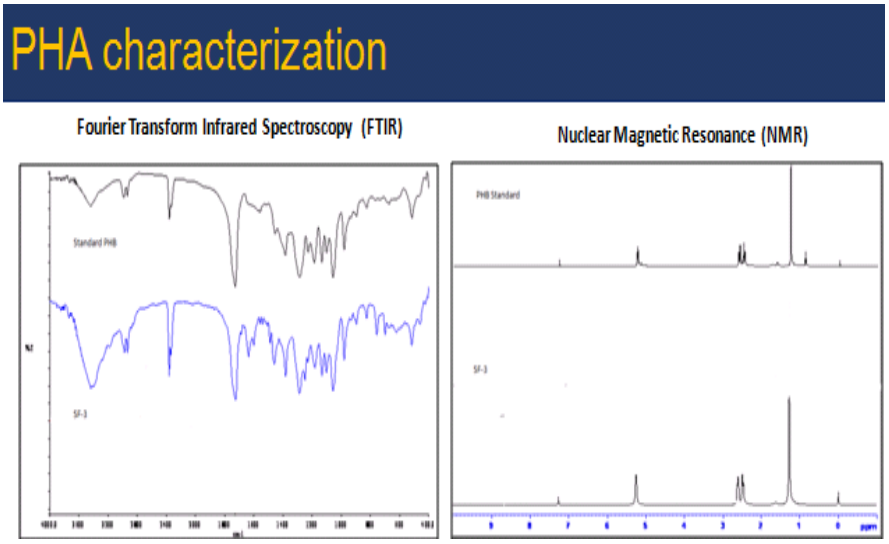

Fig. 7: Characterization of PHA

Conclusion

In this research, inexpensive raw material rice bran was used as a carbon sources to produce PHA, Various bacterial strain were isolated and screened from jaggery waste for PHB production using rice bran as a carbon source, based on their performance for PHA production, one best bacterial isolate with favorable performance was selected, it was found that rice bran not only reduce the price of material, but also improve the cell density and PHA accumulation, rice bran is potentially viable as a carbon source for commercial, great scale production of PHB.

The bacterial isolate RB2 can be considered as potential bacteria for conversion of rice bran containing starch into PHB, SF-3 or RB2 isolate utilize rice bran containing large amount of starch as sole carbon source for growth and PHB bio product accumulating PHB up to 68% and 47% (CDW) respectively, as a conclusion SF-3 (2%RB) bacteria can be considered as excellent candidate to produce industrial PHB form starch.

Authors' Contribution

All authors contributed equally in all stages of research and preparation of manuscript. Similarly, final form of manuscript was approved by all authors.

Conflict of Interest

The authors declare that there is no conflict of interest with present publication.

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