ISSN: 0976-3031

Available Online at http://www.recentscientific.com

CODEN: IJRSFP (USA)

International Journal of Recent Scientific Research
Vol. 10, Issue, 07(A), pp. 33359-33374, July, 2019

International Journal of Recent Scientific Research

DOI: 10.24327/IJRSR

Research Article

PRODUCTION AND CHARACTERIZATION OF POLYHYDROXYALKANOATES (PHA) BY *BACILLUS MEGATERIUM* STRAIN JHA USING INEXPENSIVE AGRO-INDUSTRIAL WASTES

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DOI: http://dx.doi.org/10.24327/ijrsr.2019.1007.3656

ARTICLE INFO

Article History:
Received 06th April, 2019
Received in revised form 14th May, 2019
Accepted 23rd June, 2019
Published online 28th July, 2019

Key Words:

Polyhydroxyalkanoates, Molasses, Agro-industrial wastes, *Bacillus megaterium* strain JHA.

ABSTRACT

The use of biopolymer as a substitute for synthetic plastic has been gaining importance since the last two decades. One of the challenges associated with biopolymer production is the cost involved in production which can be reduced by use of alternative carbon sources like agro-industrial waste. *Bacillus megaterium* strain JHA isolated from oil contaminated soil was analysed for its potential to accumulate PHA using glucose. The organism was screened to check for PHA accumulation using agro-industrial wastes like molasses, kakvi, baggase, banana peel, potato peel, mango peel, muskmelon peel, lychee seed, jackfruit seed, textile effluent waste, waste oil, neera, nirmalaya and protein powder. All the substrates were utilized by *Bacillus megaterium* strain JHA for PHA biosynthesis. Among the agro-industrial waste screened, molasses showed maximum PHA accumulation. On optimization of media, E2 medium (pH 8) devoid of nitrogen with 20 g% molasses showed maximum PHA accumulation of 19.52 g/l after incubation at 72 hrs at 30°C. The ability of the organism to accumulate PHA was confirmed using Nile blue A plate assay and Confocal Microscopy. The bioplastic layer formed was further characterized by HPTLC, FTIR, NMR, DSC, TGA and GPC which was confirmed to be a type of polyhydroxyalkanoate.

INTRODUCTION

From the beginning of civilization, the era has been named depending on the material used by man and thus with the end of the 20th century began the 'Polymer Age' (Wnek, 2008). The name accurately depicts the importance of plastics since it is being used in all industries universally (Raza *et al*., 2017). Polymers being adaptable can be easily moulded as per requirement, are used extensively in various fields including household, food industry, medical, packaging and transport (Wnek, 2008). One of the major problems associated with the use of plastic is its disposal since they are either not easily degraded and have a tendency to accumulate in the environment (Reddy *et al*., 2003; Keshavarz and Roy, 2010; Shenoy *et al*., 2012; Raza *et al*., 2019). These concerns have given a stimulus to develop eco-friendly plastics such as PHAs with the help of various biological systems like plant, animal and microorganism (Verlinden *et al*., 2007; Rai and Roy, 2011). Polyhydroxyalkanoates (PHAs) are a group of biodegradable, storage polyesters produced by various prokaryotic organisms particularly during nitrogen or phosphorus limitation and in the presence of excess amount of carbon (Ramsay *et al*., 1990; Steinbuchel and Schlegel, 1991; Shenoy *et al*., 2012). These polymers have properties similar to the synthetic plastics and hence are being considered as a good substitutes for petrochemical polymers like polyethylene (PE), polypropylene (PP), nylon, and polyvinyl chloride (PVC) (Rawte and Mawinkurve, 2001; Raza *et al*., 2018). Over the last two decades, a large amount of work is being carried out in order to produce economically sustainable biodegradable plastics. PHAs are being produced by various gram negative and gram positive organisms through fermentation at the laboratory or pilot scale level (Cui *et al*., 2017). The major restrictions in large scale production of the biopolymers are the low yield and cost of the carbon substrate that accounts for 50% of the manufacture cost (Choi and Lee, 1999; Kim and Chang, 2000; Shivakumar, 2012).

One of the alternatives to reduce the cost of production is by the use of agro-industrial waste such as cane molasses, corn steep liquor, whey, baggase, jackfruit seed, banana peel, orange peel, waste frying oil and waste paper as a feedstock for PHA synthesis (Gouda *et al*., 2001; Ramadas *et al*., 2009; Akaraonye *et al*., 2012; Naheed *et al*., 2012; Gowda and Shivakumar, 2014; Umesh *et al*., 2017; Vijay and Tarika, 2018; Al-Battashi

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et al., 2019; Kumar and Kim, 2019). These can substitute the commercially used carbohydrates like glucose, sucrose, lactose etc. (Steinbuchel and Fuchtenbusch, 1998; Ojumu *et al*., 2004; Gowda and Shivakumar, 2014) which in turn can reduce the production cost and also help in the treatment of the waste turning it into an economically viable by-product. The work aimed to decide the likelihood of using agro-industrial waste as a substrate for PHA production by *Bacillus megaterium* strain JHA.

MATERIAL AND METHODS

Microorganism and Preparation of Inoculums

In our previous study, PHA accumulating *Bacillus megaterium* strain JHA (Mascarenhas and Aruna, 2017) was isolated from oil contaminated soil and was used in the current work. 14 hr old growth of *Bacillus megaterium* strain JHA was used for pre-growth in 50 ml sterile nutrient broth at 30°C at 120 rpm for 24 hrs. A culture suspension of 0.2 at $O.D_{540nm}$ was prepared in sterile nutrient broth (pH 7). An inoculum size of 2.5% w/v was used for inoculation in 60 ml modified sterile E2 medium (Lageveen *et al*., 1988) complemented with 2% agro-industrial waste as carbon source (Shenoy *et al*., 2012).

Agro-Industrial Wastes

To check for the efficiency of agro-industrial waste to be used as carbon feedstock, sources were acquired from various places. Molasses, Kakvi (liquid jaggery) and baggase were obtained from the sugar factory at Kolhapur. Fruit and vegetable waste like banana peel, potato peel, mango peel, muskmelon peel, lychee seed and jackfruit seed were obtained from local market at Grant road, Mumbai. Other wastes such as textile effluent waste, waste oil (procured from local vendor used for frying eatables), neera, nirmalaya (acquired from the nearby temple at Gamdevi, Mumbai) and protein powder obtained from the local market were used to analyse for their use as a carbon source for PHA accumulation.

Processing of the Agro – Industrial waste

The agro-industrial wastes were processed before use as a carbon source as follows: 20 g% (w/v) of molasses was suspended in distilled water and sterilized at 121°C at 15 psi for 15 min. 50 g of Kakavi was suspended in 100 ml distilled water and sterilized in the similar manner as molasses. Waste oil was sterilized in the hot air oven at 160°C for 1 hr. 100 ml of the textile effluent was sterilized for use as carbon source. Vaccum packed neera (2% v/v) and protein powder packets (2 g% w/v) were used directly as carbon sources. Waste like baggase, banana peel, jackfruit seed, lychee seed, mango peel, muskmelon peel, nirmalaya, potato peel and papaya peel were dried at 50°C. A 100 g% (w/v) of the sample was prepared in distilled water and the hydrolysate obtained was passed through a muslin cloth twice. The clear hydrolysate obtained was sterilized at 15 psi for 15 min and used as carbon source for the PHA production (Ramdas *et al*., 2009; Santimano *et al*., 2009; Tamboli *et al*., 2010; Ghate *et al*., 2011; Shivakumar, 2012; Anjali *et al*., 2014; Gowda and Shivakumar, 2014; Kulkarni *et al*., 2015; Ojha and Das, 2017).

Screening of Different Agro-Industrial by-Products for Maximum PHA Accumulation

Different agro-industrial wastes were added at the concentration of 2 g% (w/v) in modified sterile E2 medium (Lageveen *et al*., 1988). The culture suspension prepared as mentioned above was inoculated in the medium and kept at 30°C under shaker conditions (120 rpm) for 72 hrs.

Estimation of Biomass

After 72 hrs, 10 ml of the culture broth was centrifuged at 10,000 rpm for 20 min. The cell pellet was further washed twice with sterile phosphate buffered saline (pH 7.2) and centrifuged at 8000 rpm for 15 min. The supernatant was discarded and the pellet was dried at 55°C for 24-48 hrs or till a stable reading of dry weight was obtained (Grothe *et al*., 1999; Pal *et al*., 2009).

Extraction and Quantification of PHA

The remaining 50 ml of the culture broth was centrifuged at 10,000 rpm for 20 min to obtain a pellet which was washed twice with sterile phosphate buffered saline (pH=7.2). The pellet was centrifuged at 8000 rpm for 12 min. It was then suspended in 10 ml of Chloroform (Loba Chemie). This mixture was vortexed and incubated at 37°C for 24 hrs. The suspension was centrifuged at 8000 rpm for 12 min and the supernatant was then poured in sterile petri plates. The chloroform was allowed to evaporate at 30°C (Phukon *et al*., 2012). The white PHA powder obtained was quantified spectrophotometrically by Slepecky and Law method (1960) wherein the PHA powder obtained was treated with 5 ml concentrated sulphuric acid and heated in a boiling water bath for 10 min. Blank was prepared by subjecting 5 ml concentrated sulphuric acid to the same treatment as test. PHA on heating with concentrated sulphuric acid depolymerized to form crotonic acid which was quantified at 235nm under UV-VIS Spectrophotometer (Agilent) against standard crotonic acid as the standard.

Calculation: (Gomaa, 2014; Yogesh *et al*., 2014)

Cell dry Weight (CDW) was Calculated as Follows

Cell dry weight (g/l) = Weight of the dried cell in tube – Weight of the empty tube.

PHA yield (%) was calculated as follows:

PHA yield % = (Weight of PHA/ Dry cell weight) × 100.

Optimization of Media Using Varying Concentration of Molasses as Carbon Source

On screening the various agro-industrial wastes, molasses was found to accumulate maximum PHA and hence, was used for further studies. A 100 g % (w/v) stock of molasses was prepared in the similar manner as mentioned earlier for the further media optimization. In order to optimize best media to achieve maximal PHA accumulation the media was prepared in the following ways (Naheed *et al*., 2012):

a. Modified Sterile E2 medium (60 ml) with varying concentrations of Molasses (2%, 5%, 10%, 20%, 30%, 40%, 50%, and 60%)

b. Sterile E2 medium (60 ml) without nitrogen sources (Microcosmic salt and KNO_3) with varying concentrations of Molasses (2%, 5%, 10%, 20%, 30%, 40%, 50%, and 60%)
c. Sterile distilled water (60 ml) with varying concentrations of Molasses (2%, 5% and 10%).

The pH of all the above media were maintained at 8. Molasses was sterilized separately and then added to the medium. The media was also supplemented with 0.2 ml of trace elements and 0.6 ml of 100mM $MgSO_4$. $7H_2O$ solution. 2.5% (v/v) culture having $O.D_{540nm}$ 0.2 was inoculated in the above various media and kept at 30°C under shaker conditions (120 rpm) for 72 hrs. The biomass was estimated and the PHA was extracted and analysed by the method discussed previously.

Open Source R software has been used to carry out the regression analysis of the above experiments.

Confirmation of the Presence of the Biopolymer

The presence of the biopolymer in the bacterial cell after growth was confirmed by fluorescence using Nile Blue A dye by Nile blue A plate assay and Confocal microscopy.

Nile blue A plate assay

Bacillus megaterium strain JHA was spot inoculated on modified sterile E2 agar medium without nitrogen source and 20g % (w/v) molasses as a carbon source (Ostle and Holt, 1982; Santimano *et al*., 2009). The Nile Blue A dye (0.5µg/ ml in DMSO) was incorporated into the solid medium. After spot inoculation the plates were incubated at 30°C for 72hrs. The plates were then exposed to ultraviolet light in the UV transilluminator (Varda Biotech) to check for orange fluorescence.

Confocal Microscopy

10 ml of the cell suspension from the medium containing molasses as carbon source was subjected to centrifugation at 8000 rpm for 12 min to obtain a cell pellet which was washed in sterile phosphate buffered saline (pH 7.2). A smear was prepared on glass slide, air dried, heat fixed and stained with 1% aqueous Nile Blue A dye stain. The slide was kept on water bath for 10 min and then washed first with 8% (v/v) acetic acid solution for 1 min, and then with distilled water. The air dried slide was observed under Confocal Microscope (Zeiss LSM T-PMT at the Dept. of Biosciences and Bioengineering, IIT Bombay) using green excitation filter (544-642 nm) (Legat *et al*., 2010; Phukon *et al*., 2011).

Film formation and Polymer Characterization and Analysis

The optimized broth was subjected to extraction by chloroform method as mentioned earlier. The chloroform layer was slowly poured in sterile petri plates and kept undisturbed for a few hours at 30°C. The chloroform was allowed to evaporate resulting in the formation of film formation (Phukon *et al*., 2012; Asad-Ur-Rehman *et al*., 2016). The film obtained was then characterized and analysed by the following methods:

High Performance thin layer Chromatography (HPTLC)

For TLC the samples, 5 µl test sample (10mg/ml) and 15 µl Std. PHB (Sigma) (1 mg/ml) were prepared in chloroform and applied on the silica gel 60 F254 (Merck) plates by the applicator. The plate was placed in the tank containing saturated mobile phase [cyclohexane: ethyl acetate: formic acid (8:2:0.1 v/v/v)]. The plate was then derivatized using Iodine crystals (Jork *et al*., 1990). The Rf value spots observed was calculated. The Rf value of the polymer obtained was compared with the standard PHB (Sigma) (Rawte *et al*., 2002; Panda *et al*., 2008; Sentilkumar *et al*., 2016). A new method of derivatization was also established for visualization of PHA spots using Anisaldehyde sulphuric acid reagent (ASR) (Sherma and Fried, 2003). The derivatized plate was scanned and visualised under white light and by using mercury lamp at 366 nm. The Rf value of the spots were then calculated. These experiments were performed at Anchrom Laboratories, Mulund using Camag HPTLC units.

FTIR Analysis

To study the different functional groups, samples were subjected to FTIR analysis. 2 mg of test sample and std. PHB (Sigma) was mixed with Potassium bromide (KBr salt) forming discs which were then analysed using FTIR (Shimadzu) which were performed at Dr. P. S. Ramanathan Advanced Instrumentation Centre at Ruia College, Mumbai (Muthazhagan and Thangaraj, 2014; Sathiyanarayanan *et al*., 2017).

NMR Analysis

The chemical structure of polymer was studied using NMR at SAIF, IIT Bombay. ^1H NMR spectra was acquired by dissolving the polymer sample in deuteron chloroform ($CDCl_3$) (Sathiyanarayanan *et al*., 2013a; Pillai *et al*., 2017).

Differential Scanning Calorimeter

The test sample (3 mg) was analysed by Differential scanning calorimeter (DSC) analysis to characterize melting temperature (Tm) and heating enthalpy (ΔH) using a DSC 8000 Perkin Elmer instrument (The Bombay textile research association, Mumbai). The test sample was heated between 30°C- 350°C under atmosphere of nitrogen at a heating range of 10°C / min and the Tm and ΔH was studied (Pillai *et al*., 2017; Sabapathy *et al*., 2019)

Thermogravimetric Analysis

The thermal stability and degradation pattern of the test samples was analysed by Thermogravimetric analysis (TGA) using TA instrument SDT Q600 (The Bombay textile research association, Mumbai). The analysis was done at a heating range of 10°C / min under an environment of nitrogen between 24°C-650°C (Ojha and Das, 2017; Pillai *et al*., 2017).

Gel Permeation Chromatography

2 mg sample was dissolved in 1 ml chloroform and analysed using Gel Permeation chromatography (GPC) to determine weight-average molecular weight (Mw), number-average molecular weight (Mn) and molecular weight distribution (Mw/ Mn). The instrument used for the analysis was Agilent 1260 Multidetector system (The Bombay textile research association, Mumbai). The GPC analysis parameters were as follows: the column used was Plgel Mixed -C with polystyrene as the internal standard (800-900000 Da), chloroform was the eluent used at the flow rate 1 ml/ min, temperature was 35° C and UV source was the detector system used. The sample (100 µl) was

injected through the instrument for analysis (Sathiyanarayanan *et al*., 2013a; Pillai *et al*., 2017).

RESULTS AND DISCUSSION

Screening of Different agro-Industrial waste for PHA Accumulation

On screening 15 different agro-industrial wastes for PHA biosynthesis, *Bacillus megaterium* strain JHA showed the ability to consume all the substrates, thus showing its versatile nature in utilization of different substrates as shown in Fig. 1. *Bacillus megaterium* strain JHA showed maximum PHA accumulation using the different agro-industrial waste in the following order Molasses > Jackfruit seed > Mango peel > Protein powder > Waste frying oil > Potato peel > Lychee seed > Papaya peel > Muskmelon peel > Neera where 6.95 g/l, 5.43 g/l, 5.42 g/l, 4.89 g/l, 3.75 g/l, 3.57 g/l, 3.47 g/l, 2.70 g/l and 2.46 g/l PHA was synthesized respectively. Minimal PHA accumulation was noted using baggase and textile waste as carbon feedstock with PHA accumulation of 0.31 g/l and 0.39 g/l respectively.

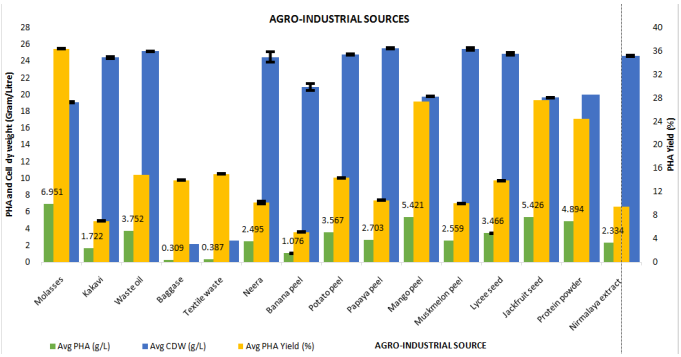


Fig 1 Screening of various agro-industrial wastes for PHA biosynthesis

The cost of the carbon substrate accounts for major expense involved in PHA accumulation and this can be significantly reduced by use of wastes generated from various industries like the agriculture and food industry (Byrom, 1987; Choi and Lee, 1997; Sudesh *et al*., 2000; Reis *et al*., 2003). PHA biosynthesis by different microorganisms from various sources such as alpechin, apple pulp waste, biodiesel liquid waste, cardboard industry effluent, crude glycerol, cooking oil, dairy waste, leguminous and food processing waste, plant oils, orange peel hydrolysate, waste effluent, waste office paper, water hyacinth and whey has been reported over the last two decades (Pozo *et al*., 2002; Koller *et al*., 2007; Pandian *et al*., 2009; Bhuwal *et al*., 2014; Preethi *et al*., 2015; Elain *et al*., 2016; Ray *et al*., 2016; Reddy *et al*., 2016; Kourmentza *et al*., 2017; Umesh *et al*., 2017; Martla *et al*., 2018; Al-Battashi *et al*., 2019; Bustamante *et al*., 2019; Rebocho *et al*., 2019). The use of waste as carbon substrate also reduces problems associated with waste disposal (Yu, 2007).

Using molasses as a carbon substrate polymer synthesis was reported in various such as *Azotobacter vinelandii* where 7.8 g/l PHA accumulation was seen (Page, 1992), recombinant *Escherichia coli* strain HMS174/pTZ18u-PHB showed 80% PHB content (Liu *et al*., 1998), *Bacillus megaterium* produced a PHA yield of 46.2% (Gouda *et al*., 2001), *Bacillus megaterium* ATCC 6748 documented 43% PHA yield (Chaijamrus and Udpuay, 2008), *Bacillus* sp. COLI/A6 showed maximum PHA yield of 54.68% (Santimano *et al*., 2009), *Enterobacter* sp. SEL2 (JF901810) and *Enterobacteriaceae* bacterium PFW1 (JF901811) showed PHA yield of 57.61±0.57% and 58.07±0.25% respectively (Naheed *et al*., 2011), maximum polymer yield of 3.64 g/l was achieved by *Bacillus cereus* SPV (Akaraonye *et al*., 2012), *Bacillus thuringiensis* IAM 12077 reported PHA yield of 23.81% (Shivakumar, 2012), *B*acillus *megaterium* strain synthesized 30.5 g/L PHA (Kanjanachumpol *et al*., 2013), *Bacillus subtilis* and *Escherichia coli* showed PHA production of 54.1% and 47.16% respectively (Gomaa, 2014), *Bacillus* sp. KSN5 produced 19.51 g/l PHA (Kalaivani and Sukumaran, 2015), *Pandoraea* sp. MA03 accumulated 0.26 g/l PHA (Coutinho de Paula *et al*., 2016), *Wickerhamomyces anomalus* VIT-NN01 showed PHA yield of 41.5% (Ojha and Das, 2017) and *Bacillus endophyticus* accumulated 10.7g/l PHA (Geethu *et al*., 2019). The results obtained in the present work also supported the fact that molasses can be used as an efficient carbon substrate enabling maximal polymer accumulation.

Maximal PHA accumulation using jackfruit seed was seen in *Bacillus sphaericus, Bacillus sphaericus* NCIM 5149, *Bacillus thuringiensis* IAM 12077, *Wickerhamomyces anomalus* VIT-NN01 and *Nocardia* sp. RD13 wherein PHA yield of 2.2 g/l, 0.690g/l, 3.93 g/l 28% and 0.34 g/l was noted respectively (Ramadas *et al*., 2009; Pandey *et al*., 2009; Gowda and Shivakumar, 2014; Ojha and Das, 2017; Deepa and Vidhya, 2018). Likewise the use of mango peel as a feedstock for PHA biosynthesis was studied in *Bacillus thuringiensis* IAM 12077 showing maximum PHA accumulation of 4.03 g/l (Gowda and Shivakumar, 2014). *Bacillus megaterium* strain JHA shows more PHA accumulation using jackfruit seed and mango peel making them the second best substrate for PHA accumulation. Further optimization needs to be done inorder to increase their efficiency as feedstock.

One of the major wastes from fast food industries is waste frying oil which has been used as an appropriate substrate for PHA production (Hori *et al*., 2002; Haba *et al*., 2007). Using this waste, maximum PHA accumulation has been reported in *Pseudomonas aeruginosa* 47T2, *Pseudomonas aeruginosa, Cupriavidus necator*, *Pandoraea* sp. MA03, *Pseudomonas aeruginosa* (KF270353), *Klebsiella pneumoniae* (STN-7), *Bacillus subtilis* (STN-8) and *Paracoccus* sp. LL1 (Vidal-Mas *et al*., 2001; Haba *et al*., 2007; Verlinden *et al*., 2011; Coutinho de Paula *et al*., 2016; Tufail *et al*., 2017; Kumar and Kim, 2019).

Potato starch and potato peel as substrate for polymer accumulation was seen in *Ralstonia eutropha* NCIMB 11599, *Bacillus sphaericus* NCIM 5149, *Bacillus thuringiensis* IAM 12077 and *Wickerhamomyces anomalus* VIT-NN01 recording maximum PHA of 94g/l, 0.710g/l, 7.4% and 30.7% respectively (Haas *et al*., 2008; Pandey *et al*., 2009; Gowda and Shivakumar, 2014; Ojha and Das, 2017). Using neera as carbon substrate for polymer accumulation, *Bacillus subtilis* and *Bacillus cereus* reported 0.284 g/l and 0.152 g/l of PHA (Ghate *et al*., 2011). The use of banana peel as a feedstock for PHA accumulation has been reported in *Halomonas campisalis* MCM B-1027,*Geobacillus stearothermophilus* R- 35646, *Cupriavidus necator*, *Bacillus siamensis* PD- A10 and

Staphylococcus aureus JH1 (Kulkarni *et al*., 2010; Vijay and Tarika, 2018).

Another waste from the sugarcane industry is baggase which has also been used as a carbon feedstock for PHA biosynthesis by *Burkholderia cepacia*, *Burkholderia sacchari* IPT101, *Halomonas campisalis* MCM B-1027, *Bacillus megaterium, Bacillus thuringiensis* IAM 12077and *Wickerhamomyces anomalus* VIT-NN01 showing PHA yield of 62%, 53%, 47% , 0.199g/l, 0.09g/l, 1.26 g/l and 27.3% respectively (Silva *et al*., 2004; Kulkarni *et al*., 2010; Ghate *et al*., 2011; Shivakumar, 2012; Gowda and Shivakumar, 2014; Ojha and Das, 2017). Textile waste-water has also been used by *Sphingobacterium* sp. ATM for PHS accumulation where 66% PHA yield has been detected (Tamboli *et al*., 2010). PHA biosynthesis using papaya peel, muskmelon peel, lychee seed, nirmalaya extract, kakvi and protein powder as substrate has been reported for the first time in the present work.

Optimization of Media and Molasses Concentration for Maximum PHA Accumulation

On screening different agro-industrial wastes, molasses was confirmed as an ideal carbon substrate for PHA production. In order to increase PHA yield different variations in media composition and molasses concentration was done to assess the media for efficient PHA production. On growing *Bacillus megaterium* strain JHA on E2 medium with varying concentrations of molasses, the isolate is capable of accumulating PHA from minimum 2% molasses to 60% molasses. The maximum PHA of 14.75 g/l and PHA yield of 55.05% was achieved using 20% Molasses as carbon source as seen in Fig. 2. The regression analysis also confirmed that with increase in molasses concentration the PHA accumulation increases.

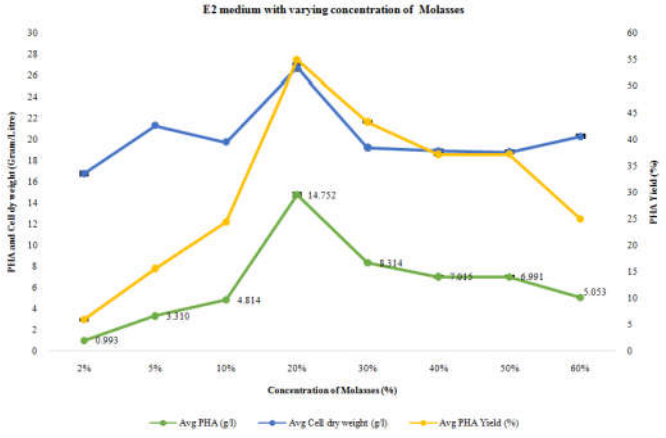


Fig 2 PHA accumulation by *Bacillus megaterium* strain JHA on E2 medium with varying concentrations of Molasses

Bacillus megaterium strain JHA was grown in production E2 medium devoid of the nitrogen sources (microcosmic salt and KNO_3) and varying concentrations of the molasses. PHA accumulation was observed in the medium in spite of nitrogen being absent in the medium. Maximum PHA accumulation of 19.52 g/l of PHA with 60% PHA yield was observed with 20% molasses concentration as seen in Fig. 3. The molasses is obtained as a crude product after the process of sugar extraction. It contains large number of impurities and nitrogen sources too in small concentrations. As a result a high carbon nitrogen ratio is maintained in the medium making the condition suitable for *Bacillus megaterium* strain JHA to accumulate maximum PHA. The amount of PHA recorded is reasonably higher than the PHA produced in the presence of glucose and nitrogen sources like microcosmic salt and KNO_3. Thus this medium can be used for large scale PHA accumulation. The regression analysis stated that with increase in molasses concentration in medium devoid of nitrogen, the PHA accumulation increases.

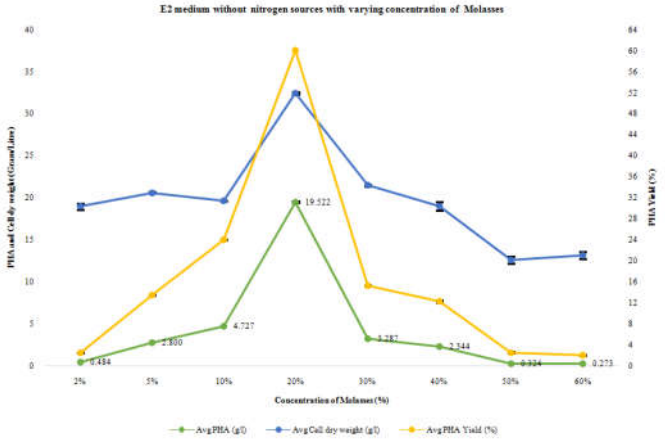


Fig 3 PHA accumulation by *Bacillus megaterium* strain JHA on E2 medium devoid of nitrogen with varying concentrations of Molasses

Bacillus megaterium strain JHA when grown in distilled water with varying concentrations of molasses showed PHA accumulation. Maximum PHA accumulation of 4.77g/l was observed with 10% molasses as seen in Fig. 4. But the amount of PHA accumulated is very less as compared to the other media variations tried earlier. It can be concluded that certain components like phosphates present in the earlier media may play a significant role in the growth of the isolate and PHA accumulation.

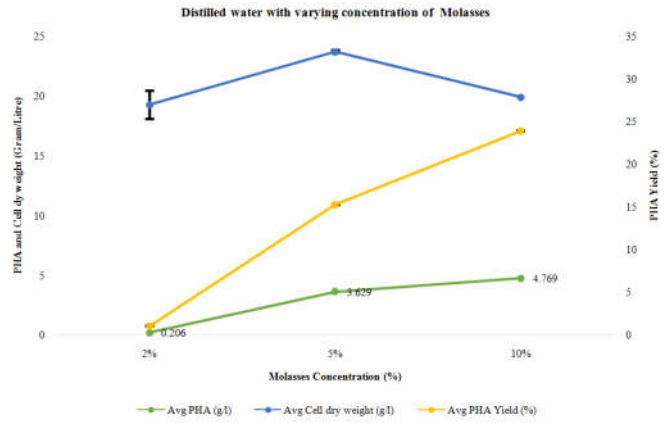


Fig 4 PHA accumulation by *Bacillus megaterium* strain JHA in distilled water with varying concentrations of Molasses

PHA biosynthesis in microbial cell is enhanced under conditions of nitrogen or phosphate limitation in combination with excess carbon substrate (Koller, 2019). *Azotobacter vinelandii* (Page *et al*., 1992) has reported maximum PHA accumulation of 23 g/l using molasses as carbon source. A setup similar to the one used in the present work was studied in *Bacillus megaterium* BA-019 where polymer biosynthesis was studied in the presence of 20 g/l molasses in the presence and absence of nitrogen source (Kulpreecha *et al*., 2009). They

observed maximum PHA yield of 55.46% when the medium was supplemented with a nitrogen source like urea. Different strains of *Bacillus megaterium* have shown their ability to utilise molasses showing maximum PHA accumulation of 1.27 g/l PHA using 3% molasses and PHA yield of 43% using 4% molasses as a carbon feedstock (Gouda *et al*., 2001; Chaijamrus and Udpuay, 2008). With increase in molasses concentration the growth of *Enterobacter* bacterium PFW1and *Enterobacter* sp.SEL2 was found to be better (Naheed *et al*., 2011). With 2 g% molasses in the production medium, PHA yield of 61.07% was reported in *Bacillus cereus* SPV (Akaraonye *et al*., 2012) whereas *Bacillus cereus* RCL 02 showed 7.24 g/l PHA accumulation (Das *et al*., 2017). Similarly PHA accumulation has been reported in different strain of *Bacillus subtilis* where PHA accumulation of 2.5 g/l, 54.1% and 15 g/l has been reported using 10g% and 6g% molasses (Gomaa, 2014; Nair *et al*., 2014). Maximum PHA yield 47.16% was documented in *Escherichia coli* with 8% molasses (Gomaa, 2014). *Wickerhamomyces anomalus* VIT-NN01, *Acinetobacter nosocomialis* RR20A and *Bacillus endophyticus* showed PHA accumulation 19.50 g/l, 3.48 g/l and 10.7 g/l with 35g/l, 30 g/l and 40 g/l molasses respectively (Ojha and Das, 2017; Reddy *et al*., 2018; Geethu *et al*., 2019). The present study too confirms that molasses concentration can influence polymer biosynthesis. Sugar cane molasses is a viscous, sticky fluid obtained after extraction of sugar from sugarcane (Naheed *et al*., 2011; Shasaltaneh *et al*., 2013). Molasses being inedible is regularly used as an animal feed supplement (Wang *et al*., 1979). The molasses produced also has a negative impact on the environment and is generally converted to fusel oil before disposal (Akaraonye *et al*., 2012). Sugar cane molasses is rich in sugars like sucrose, glucose, fructose, trace elements like calcium, iron, magnesium and potassium and vitamins such as niacin, pyridoxine, riboflavin and thiamine which can act as growth factors permitting microbial growth (White, 1954; Malathi and Chakraborty, 1991; Rodrigues *et al*., 2006; Albuquerque *et al*., 2007; Berwanger *et al*., 2007; Shasaltaneh *et al*., 2013). The ability of the organism to synthesize PHA using molasses is due to the highly unbalance C:N ratio in the production medium causing repression of action of the enzymes involved in the TCA cycle and the production of excess Acetyl Co-A which in turn is used for polymer synthesis (Dawes and Senior, 1973; Saranya and Shenbagarathai, 2011). The capability of *Bacillus megaterium* strain JHA to accumulate PHA using a high concentration of molasses (20 g %) in a medium devoid of nitrogen provides a very good alternative to bioplastic production that too at a low cost making the organism ideal for production of an economically valuable product.

Confirmation of PHA Accumulation

Bacillus megaterium strain JHA showed orange fluorescence on exposure to UV light when grown on plate containing E2 medium devoid of nitrogen with 20 g % molasses as seen in Fig. 5. On observation of the bacterial smears stained using Nile blue A dye under the Confocal Microscope (Zeiss LSM T-PMT), the bacterial cells appeared as glowing fluorescent bodies due to the polymer accumulation. Fig. 6 represents the fluorescent cells of *Bacillus megaterium* strain JHA seen under the confocal microscope. Thus the Nile blue A plate assay and Confocal microscopy confirmed the ability of *Bacillus megaterium* strain JHA to accumulate PHA as inclusion bodies using molasses as a carbon source and under nitrogen limited conditions.

Ostle and Holt (1982) confirmed the use of Nile Blue A dye for PHA detection since it does not stain other inclusion bodies like glycogen and polyphosphate. It has a high affinity for PHA and produces an orange fluorescence on exposure to utraviolet light. The Nile blue A plate assay was devised by Kitamura and Doi (1994) to screen for PHA accumulation in various organisms which was then modified by Spiekermann *et al*. (1999) where small quantities of the dye was directly incorporated into the production medium itself for superior detection. Santimano *et al*. (2009) used the plate assay method as a qualitative method to screen different agro-industrial wastes to be used as carbon feedstock for PHA accumulation in *Bacillus* strain COL1/6 species. The Nile blue A plate assay method has reportedly been widely used for detection of PHA granules within the bacterial cells (Rawte *et al*., 2002; Berlanga *et al*. 2006; Legat *et al*., 2010; Shenoy *et al*., 2012; Subin *et al*., 2013; Wagle *et al*., 2016).

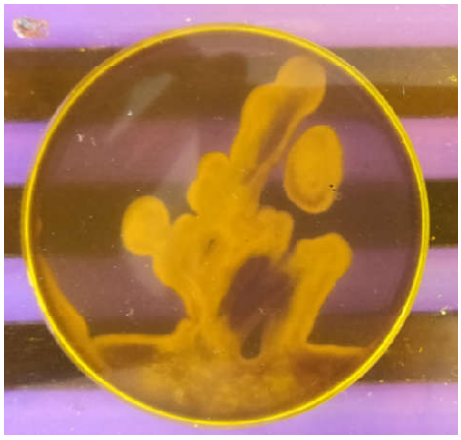
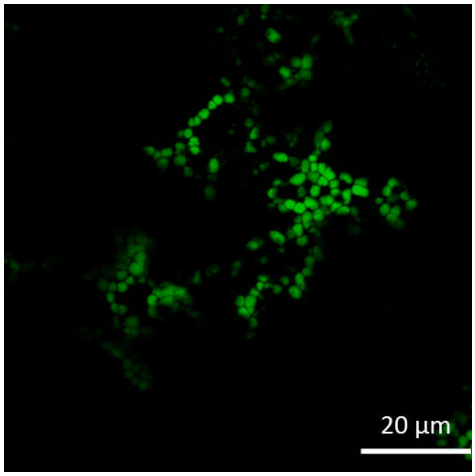
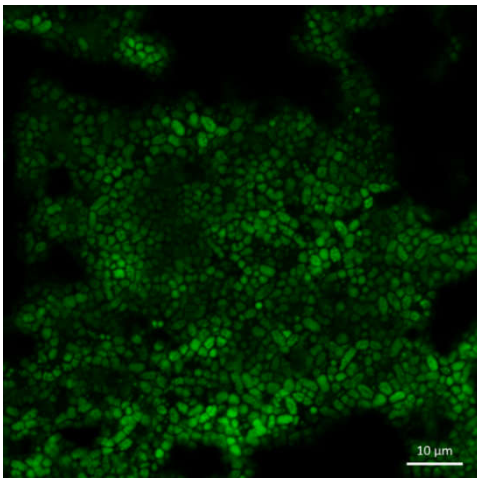


Fig 5 *Bacillus megaterium* strain JHA showing orange fluorescence in the presence of molasses by the Nile Blue A plate assay



A



B

Fig 6 *Bacillus megaterium* strain JHA showing green fluorescence (green filter used) in the presence of molasses under the confocal microscope A) thin smear B) thick smear

Legat *et al*. (2010) confirmed the affinity of Nile Blue A dye to intracellular PHB and observed superior images of *Halococcus* species against high contrast. By using the confocal microscope, the PHA granules can be detected quickly and easily. The Nile blue A staining method has been used as a reliable indicator to quantitate and estimate the amount of PHA in *Bacillus megaterium* cells (McCool *et al*., 1996) whereas Fradinho *et al*. (2013) used the Nile blue A staining method to identify bacterial cells containing PHA inclusion bodies from a mixed photosynthetic culture. The Nile blue A stained cells of recombinant *Escherichia coli* strain (K24K), *Bacillus circulans* MTCC 8167, *Bacillus thuringiensis* GVP, *Paracoccus* sp. LL1 were observed under fluorescent microscope (Nikel *et al*., 2006; Phukon *et al*., 2011; Charen *et al*., 2014; Kumar and Kim, 2019).

Film formation

During extraction process, the bacterial cells were placed in chloroform and incubated for 24 hrs. After the incubation period, the cells were subjected to centrifugation and the supernatant containing chloroform was placed in sterile petri plates allowing the chloroform to evaporate at 30° C. The chloroform dissolves the PHA present in the bacterial cells. Thus on evaporation, of the chloroform, a white film was observed as observed in Fig. 7. On visualization the film appeared to be smooth and glossy. It appeared brittle at certain areas which may be due to fewer amounts of PHA granules.

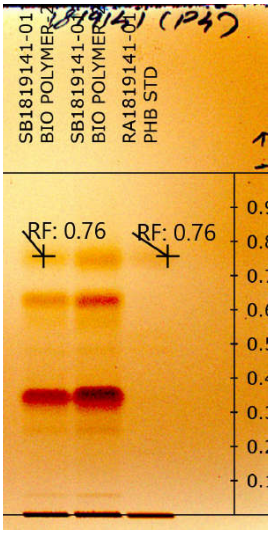
Fig 7 Physical appearance of the polymer film obtained from *Bacillus megaterium* strain JHA

A thin PHA layer was observed in *Alcaligenes eutrophus* MTCC 1285, *Azotobacter beijerinickii* , *Cupriavidus necator, Halomonas campisalis* MCM B-1027 and *Acinetobacter junii* BP 25 after extraction with chloroform (Kumar and Prabakaran, 2006; Prabhu and Murugesan, 2010; Jain and Tiwari, 2015; Kulkarni *et al*., 2015; Sabapathy *et al*., 2019). Shalin *et al*. (2014) obtained a brittle PHA film from *Bacillus firmus* NII 0830 whereas Asad-Ur-Rehman *et al*. (2016) obtained a white PHB film without cracks from *Bacillus cereus* NRRL-B-3711. It was concluded that the film formation may be due to the interlinking of the PHB granules with each other (Kumar and Prabakaran, 2006; Prabhu and Murugesan, 2010).

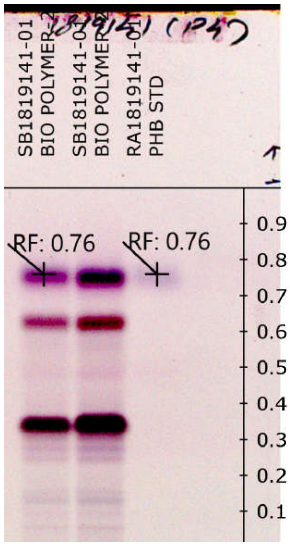
Analysis of the Polymer

HPTLC Analysis

On comparing the HPTLC of the biopolymer extracted from *Bacillus megaterium* strain JHA with standard PHB (Sigma) molecule, Rf value of the PHA was calculated as 0.76 was found to be similar to the Rf value obtained of standard. This confirmed that the extracted polymer was a PHB derivative. Fig 8 A shows brown spots on derivatization of the TLC plate using iodine vapours while Fig. 8 B shows purple spots on derivatization of TLC plates using Anisaldehyde sulphuric acid reagent (ASR). On derivatization three distinct spots were observed in the test sample as compared to only one spot in the standard PHB sample. When the TLC plates were exposed to iodine vapours, yellow brown spots were observed (Rawte *et al*., 2002; Girdhar *et al*., 2014; Preethi *et al*., 2015).The PHB recovered from *Staphylococcus epidermidis* showed an Rf value of 0.71 on using a mobile phase of ethyl acetate and benzene (Marjadi and Dharaiya, 2014).



A



B

Fig 8 HPTLC of the biopolymer and Standard PHB (Sigma). The plates were derivatized A) Iodine vapours B) Anisaldehyde sulphuric acid reagent (ASR)

On performing TLC on esters of PHA extracted from five different isolates, Rawte and Mavinkurve (2002) observed two distinct bands having Rf values 0.75 and 0.6 and a variety in the band pattern thus commenting on the diversity of polymer accumulation in microbes. The band having Rf value of 0.75 was concluded to be of non-polar, dimeric hydroxybutyrate and has the tendency to separate first (Abe *et al*., 1994; Rawte and Mavinkurve, 2002; Panda *et al*., 2008; Girdhar *et al*., 2014). Thus, it can be concluded that the type of carbon substrate available to the bacteria, influences the chemical composition of the PHA (Brandl *et al*., 1988; Lageveen *et al*. 1988; Gross *et al*., 1989). All these observations support the results obtained in the present study, thus conforming the polymer extracted from *Bacillus megaterium* strain JHA to be a PHA. The use of ASR for derivatization has been reported for the first time in the present work. Also the mobile phase used in the present work is different from the one cited in the literature.

FTIR Analysis

To determine the functional groups present in the extracted biopolymer from *Bacillus megaterium* strain JHA, the PHA film was subjected to FTIR analysis. The different bands observed on analysis were depicted in Fig. 9. The biopolymer showed prominent bands at 2935cm^{-1} and 2920 cm^{-1} which resemble the C-H stretching vibrations of the methyl group (Bhuwal *et al*., 2014; Senthilkumar *et al*., 2016). The prominent peak at 1719 cm^{-1} specifies the carbonyl stretching of the ester group (C=O) (Bhuwal *et al*., 2014; Kovalcik *et al*., 2017). Additional bands corresponding to the blending of the –CH bond in CH_2 and CH_3 groups were observed at 1449 cm^{-1} and 1382 cm^{-1} respectively (Reddy *et al*., 2015; Rodrigues-Contreras *et al*., 2015; Sathiyanarayan *et al*., 2013a; Bhatia *et al*., 2019). Series of bands between 1300 cm^{-1} -1250 cm^{-1} confirm the presence of C-O bond of the ester group (Reddy *et al*., 2015; Asad-Ur-Rehman *et al*., 2016). The presence of C=O and C-O functional group confirms the structure of polyhydroxybutyrate. The results of the extracted PHA from *Bacillus megaterium* strain JHA closely resembled the results of the standard PHB molecule thus confirming the structure of polyhydroxyalkanoate to be a type of PHB.

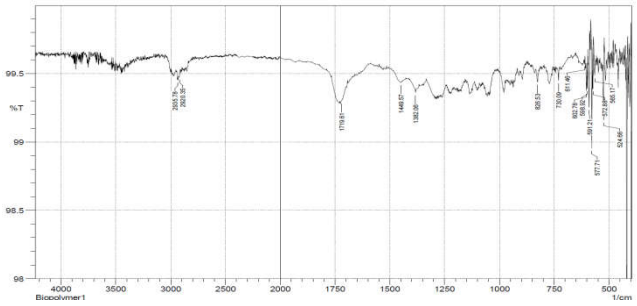


Fig 9 FTIR Analysis of PHA extracted from *Bacillus megaterium* strain JHA

FTIR has been considered as an essential tool enabling the study of microorganisms and their cell components even in their original form (Hong *et al*., 1999). FTIR being a sensitive tool has been extensively used to reveal the conformational deviations of macromolecules and has been applied to study PHA qualitatively (Xu *et al*., 2002). The polymer extracted from *Bacillus cereus* NRRL-B-3711 showed prominent bands at 1728 cm^{-1} and 1284 cm^{-1} which corresponds to C=O and C-O group respectively (Asad-Ur-Rehman *et al*., 2016). Similar results were reported by Reddy *et al*. (2015) on analysing the PHA extracted from *Bacillus* sp. CYR1 by FTIR showing the C-H and carbonyl bonds as standard PHB. On FTIR analysis of the PHA obtained from *Synechocystis salina* stretching of the CH3 group was observed at 1453 cm^{-1} and 1379 cm^{-1} (Kovalcik *et al*., 2017). The bioplastic produced by *Bacillus circulans* MTCC 8167 strain exhibited the C-H and carbonyl stretching bands like standard PHA at 1735 cm^{-1} and 1206 cm^{-1} respectively (Phukon *et al*., 2012).

NMR Analysis

The ^{1}H NMR spectrum of the extracted biopolymer from *Bacillus megaterium* strain JHA was performed to analyse its chemical structure and primary sequence of the PHA. Based on their peak positions observed in Fig. 10, the protons showed the presence of characteristic signals. A doublet was observed at 1.27 ppm attributed to the methyl group coupled to one proton, a doublet of a quadruplet is seen at 2.47 ppm which is a characteristic of methylene group, a multiplet at 5.25 ppm represents the methyne group. Two broad peaks were seen one at 1.04 ppm and another between 7.2 -7.3 ppm which are a characteristic of residual chloroform. Thus it can be concluded that the biopolymer obtained is in the form of polyhydroxybutyrate (PHB). The above outcomes corroborated with the results obtained on analysing PHA from *Bacillus* sp. NA10 (Bhuwal *et al*., 2014), Cupriavidus *necator* and *Burkholderia sacchari* (Rodrigues-Contreras *et al*., 2015), *Bacillus* sp. CYR1 (Reddy *et al*., 2015), *Bacillus cereus* PW3A (Babruwad *et al*., 2015), *Bacillus aryabhattai* (Pillai *et al*., 2017), *Bacillus megaterium* (Pradhan *et al*., 2018) and *Cupriavidus necator* H16 (Koller *et al*., 2018).

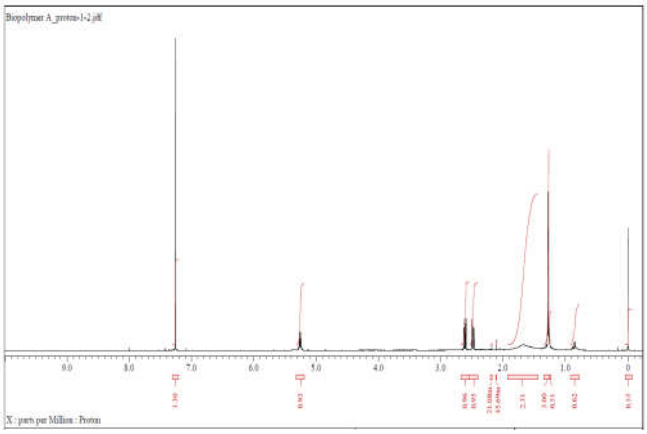


Fig 10 ^{1}H NMR analysis of PHA extracted from *Bacillus megaterium* strain JHA

DSC Analysis

Differential scanning calorimetry analysis helps in understanding the thermal transitions taking place when the polymer is heated. Through the DSC analysis it was observed that the polymer obtained from *Bacillus megaterium* strain JHA showed two peaks with the melting temperature of (Tm) at 136.41°C and 158.04°C and ΔH of 7.21 J/g and 32.50 J/g respectively as shown in Fig. 11. On observing various peaks, it can be concluded that the PHA extracted from *Bacillus megaterium* strain JHA contains a combination of different PHA fractions.

Asad-Ur-Rehman *et al*. (2016) stated that understanding the thermal characteristics of PHA is essential since it provides information regarding polymer stability, phase changes and polymer shelf life. Low thermal properties may be due to presence of impurities or maybe a property of the polymer synthesized by the organism (Rodriguez-Contreras *et al*., 2013). Different methods of fermentation and extraction procedures can influence the thermal properties of the extracted biopolymer (Valappil *et al*., 2007; Rodriguez-Contreras *et al*., 2013; Singh *et al*., 2013).

Multiple Tm peaks have been reported by Rodriguez-Contreras *et al*. (2013) on performing DSC analysis on the PHA extracted using chloroform in *Bacillus megaterium* strain uyuni S29. They reported Tm value at the third peak as 161°C which is quite close to the results obtained in the present study. They discussed that the presence of multiple peaks indicates different polymer fractions which show different melting points due to different rates of degradation. Thus, confirming that the synthesized PHB is a blend of various fractions with different molar masses. The DSC thermogram of PHA extracted from *Natrinema ajinwuensis* RM-G10 showed two melting temperatures at 143°C and 157.7°C (Mahansarai *et al*., 2018). The occurrence of two melting temperatures confirmed the occurrence of two monomer groups in the biopolymer (Mitomo *et al*., 1999; Don *et al*., 2006). Koller *et al*. (2008) observed two melting endotherms Tm1 and Tm2 at 149.7°C and 160.7°C respectively in the PHA extracted from osmophilic microorganism. They concluded that low Tm values are essential for polymer processing and it may be due to presence of 3-HV units or polymer blends. The presence of 3-HV may lead to an interruption of the very crystalline PHB matrix (Doi, 1990) and the polymer formed may be concluded to be heterogeneous in nature (Koller *et al*., 2008). A similar trend was observed in the present analysis. The melting temperature reported from the PHB extracted from *Bacillus cereus* SPV and *Bacillus aerophilus* RSL- 7 is 160.83°C and 164°C respectively (Valappil *et al*., 2007; Sabapathy *et al*., 2019). These results are quite close to the one reported in the present study.

The melting temperature of PHA produced by *Alcaligenes latus* was 151.46°C which was lower as compared to the present study (Wang *et al*., 2013). The melting temperature and ΔHm of the PHA obtained from *Dinoroseobacter* sp. JL1447 was 175.8°C and 51.87 J/ g respectively (Xiao and Jiao, 2011) whereas the DSC thermogram of the polymer from *Bacillus subtilis* NG05 showed a Tm of 132.54°C (Singh *et al*., 2013). Chaijamrus and Udpuay (2008) reported the melting temperature and ΔH of commercial PHB as 172.1°C and 90.2 J/g respectively which is quite high from the above obtained results. A lower Tm value was observed in the PHA obtained from recombinant *Escherichia coli* grown on molasses as compared to the one grown on sucrose. Thus by incorporation of molasses in the production media an increase in the addition of the longer side chain in the polymer was observed (Saranya and Shenbagarathai, 2011).

The PHB when present within microbial cell appears amorphous in nature but becomes crystalline on extraction with various solvents and free-dry techniques (Hahn *et al*., 1995). The crystalline nature of the polymer can be calculated by comparing ΔH of the polymer with the ΔH of 100% crystalline PHB which has been reported as 146.37 J/g (Barham *et al*., 1984; Valappil *et al*. 2007; Pradhan *et al*., 2018). From table 1, it can be observed that the degree of crystallinity for the PHA extracted from *Bacillus megaterium* strain JHA was 20.42 %. These results are quite similar to the Xc of 23% observed by Pradhan *et al*. (2018) in *Bacillus megaterium*. The melting point, ΔH and Xc values for PHA extracted from *Burkholderia cepacia* IPT 438 was 157.4°C, 56 J/g and 38.4% respectively whereas *Cupriavidus necator* IPT 027 was 175.9°C, 84.4 J/g and 57.8% respectively (Ribeiro *et al*., 2015). As the crystalline nature of the polymer decreases, the brittle character of the polymer reduces thus it favours processing of the biopolymer (Gunaratne and Shanks, 2005; Pradhan *et al*., 2018).

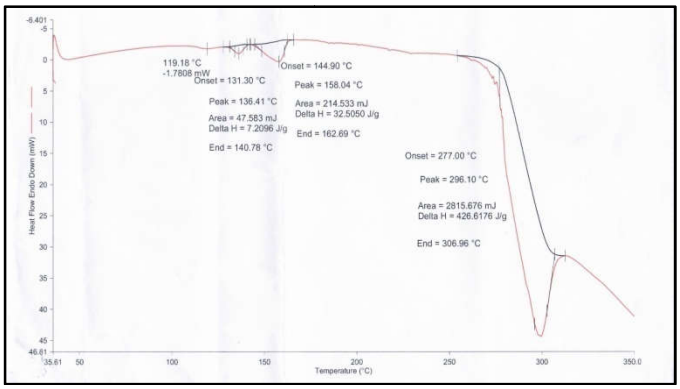


Fig 11 Thermograms of DSC and TGA analysis of PHA obtained from *Bacillus megaterium* strain JHA

Thermogravimetric Analysis

The thermal degradation of the biopolymer film extracted from *Bacillus megaterium* strain JHA occurs at 277°C with 98.04% loss at 296.10°C as in Fig. 11. The thermal stability of the

extracted polymer was higher that standard PHB (Sigma) which was reported as 266°C- 272 °C (Xiao and Jiao, 2011)

Higher thermal degradation as compared to standard has been reported in the PHA extracted from *Bacillus sphaericus* NII 0838 showed maximum decomposition at 291°C (Sindhu *et al*., 2011), *Dinoroseobacter* sp. JL1447 at 285°C (Xiao and Jiao, 2011), *Staphylococcus epidermidis* at 296.91°C (Marjadi and Dharaiya, 2014), thermal degradation in *Burkholderia cepacia* IPT 438 and *Cupriavidus necator* IPT 027 was observed at 301.5°C and 291.6°C respectively (Ribeiro *et al*., 2015), thermal degradation in *Bacillus aryabhattai* PHB10 occurs between 287°C with 96.08% loss of mass (Pillai *et al*., 2017), *Cupriavidus necator* IPT 026 showed a maximum Td of 334.42°C and weight loss of 99.35 % (Rodrigues and Druzian, 2018), *Burkholderia sacchari* at 301.5°C (Al-Battashi *et al*., 2019) and *Bacillus aerophilus* RSL-7 at 276.21°C (Sabapathy *et al*., 2019). These observations support the present work.

Lower Td has been observed in the polymer extracted from *Wickerhamomyces anomalus* VIT-NN01 250-255°C of 88% (Ojha and Das, 2017) while in *Burkholderia cepacia* ATCC 17759 Td increased from 268.6°C to 281.5°C in xylose and glycerol based medium (Zhu *et al*., 2010). The major temperature of decomposition for the PHA is found to be linked with the ester cleavage of PHA component by elimination reaction (Ojha and Das, 2017). For polymer moulding, maximum difference is required between melting temperature and higher degradation temperature (Zhu *et al*., 2010; Sathiyanarayan *et al*., 2017). The crystalline nature of a PHA influences polymer degradation wherein the amorphous regions degrade faster as compared to the crystalline regions (Iannace *et al*. 2001).

Gel Permeation Chromatography Analysis

The molecular weight distribution of the polymer was determined using GPC. On performing the GPC analysis, the chromatogram shows the presence of two peaks. The first peak reported the M_N = 47.84 kDa and M_W= 71.65 kDa with a polydispersity index of 1.49 while the second peak reported the M_N = 0.08 kDa and M_W= 0.55 kDa with a polydispersity index of 6.85 as in table 1. The presence of two peaks confirmed the results obtained from DSC analysis that the biopolymer obtained contains PHB blends. The molecular weight of the PHA is influenced by the type of carbon source used as substrate (Yamane *et al*., 1996; Chanprateep *et al*., 2010). In *Bacillus megaterium* uyuni S29 on observing the GPC chromatogram showed the presence of two main peaks corresponding to two different molar masses at 600 kDa and 125 kDa and having a polydispersity index of 1.2 and 1.5 respectively. On the basis of the results of DSC and GPC analysis, it was concluded that the polymer extracted contained a blend of different PHB fractions (Rodriguez-Contreras *et al*., 2013). These observations corroborated with the results in the present study. Lundgren *et al*. (1965) commented that mostly the end groups act as impurities containing lower molar masses which melt at a low temperature.

The M_N and M_W in *Bacillus cereus* SPV was found to be 339 kDa and 885 kDa respectively (Valappil *et al*., 2007), whereas in *Bacillus* sp. INT005, it was reported to be 281kDa and 525kDa respectively (Tajima *et al*., 2003). Both showed a polydispersity index of 2.6. On performing GPC analysis the M_N, M_W and PDI values of the PHA extracted from *Bacillus megaterium* MSBN04 was reported as 390 kDa, 670kDa and 1.71 respectively (Sathiyanarayan *et al*., 2013a) whereas the PHA extracted from *Bacillus licheniformis* MSBN12 showed 1600kDa, 2900kDa and 1.75 respectively (Sathiyanarayan *et al*.,2013b). Low values of M_N, M_W and polydispersity index of 39.15 Da, 54.78 Da and 1.4 respectively was observed on performing GPC analysis on the polymer extracted from *Pseudomonas aeruginosa* NCIB 40045 (Fernandez *et al*., 2005). The polymer extracted from *Bacillus circulans* MTCC 8167 showed polydispersity index of 1.21 (Phukon *et al*., 2012) whereas the polymer from *Pseudomonas putida* strain KT2047A showed the PDI value as 1.24 (Ma *et al*., 2009). These results are quite similar to the one in the present study. Phukon *et al*. (2012) stated that the polymers showing low PDI values can be used for commercial purpose without much processing in order to bring uniformity in the chain length.

Table 1 Summary of FTIR, ^{1}H NMR, TGA, DSC and GPC analysis of the biopolymer extracted from *Bacillus megaterium* strain JHA

Polymer Analysis		Group or Moiety	PHA from *Bacillus megaterium* strain JHA
FTIR		-CH	2935 cm^{-1} , 2920 cm^{-1}
		-C=O	1719 cm^{-1}
		$-CH_2$	1449 cm^{-1}
		$-CH_3$	1382 cm^{-1}
		-C-O	1300-1250 cm^{-1}
^{1}H NMR		-CH	5.25 ppm
		$-CH_2$	2.47 ppm
		$-CH_3$	1.27 ppm
DSC	First peak	Tm (°C)	136.41
		ΔH (J/g)	7.21
		Xc (%)	4.83
	Second peak	Tm (°C)	158.04
		ΔH (J/g)	30.50
		Xc (%)	20.42
TGA		Td (°C)	296.10
GPC	First peak	M_N (kDa)	47.84
		M_W (kDa)	71.65
		PDI (M_W/M_N)	1.49
	Second peak	M_N (kDa)	0.08
		M_W (kDa)	0.55
		PDI (M_W/M_N)	6.85

CONCLUSION

One of the major hindrances in production of bioplastic is the cost involved in its production. The aim of this study was to find a suitable alternative to reduce cost. On screening fifteen different agro-industrial wastes, molasses (2 g %) was found to be an ideal carbon substrate for PHA accumulation by *Bacillus megaterium* strain JHA. Using molasses as carbon feedstock, the medium and concentration was optimized to increase PHA yield. *Bacillus megaterium* strain JHA showed maximum PHA accumulation of 19.52 g/l with PHA yield of 60.02% using E2 medium devoid of nitrogen sources and 20 g% molasses at 30° C after 72 hrs of incubation. The Nile Blue A plate assay and confocal microscopy confirmed the ability of *Bacillus megaterium* strain JHA to accumulate PHA. On extraction a biopolymer film was also obtained which was characterized further. The HPTLC, FTIR, NMR analysis confirm that the extract biopolymer is a type of polyhydroxybutyrate. The melting temperature, decomposition temperature was determined by DSC and TGA analysis and the polymer was

found to be thermally stable. GPC analysis showed low polydispersity index, thus the film could be used without much processing. Molasses is the major waste obtained from the sugar industry. India being the second largest country in sugar production, the waste could be substantially be used for polymer synthesis. As a result, the problem associated with waste disposal can also be countered.

Acknowledgement

The authors wish to thank the University Grants Commission, Govt. of India (UGC MINOR GRANT) for providing with financial assistance during the course of the present work.

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