Screening, Isolation, and Characterization of PHB producing bacteria and Biosynthesis of PHB using different wastes materials.

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ABSTRAC: The environmental impacts caused by plastics wastes have attracted worldwide concern. Bacteria can synthesize a wide range of bio-polymers that serve diverse biological function and have material properties suitable for numerous industrial and medical applications. Polyhydroxyalkanotes are polymers produced by bacteria, among which polyhydroxybutyrates are one major group. Since the production of bioplastics is expensive so many techniques have been adopted for its large scale production. The following study was undertaken to screen and isolate bacteria producing such compounds. Different soil and marine water samples were collected and streaked onto various agar media for isolation. Colonies showing different morphologies were selected for further studies. Their morphological biochemical characteristics were studied. The bacteria were then analyzed for the PHB granules by Carbol fuchsin and Sudan black staining. And for conformation bacteria were also cultivated in presence of Nile blue staining. The colonies that showed fluorescence were selected and were inoculated in production media containing organic wastes for the production of bio-plastics.

Key words: 1. Biopolymers 2. Polyhydroxyalkanotes3. Bioplastics 4. fluorescence

1. Introduction.

The exponential growth of the human population has led to the accumulation of huge amount of non-degradable waste materials across our planet. Living conditions in the environment are therefore changing in such a way that the presence of non-biodegradable deposit is affecting the possible survival of many species (Kumaravel *et al.,2010)*.. The toughness of the disposal plastic has caused many serious environment problems (Bhat *et al,*2017*)*. Every activity in modern life has been subjective by plastics and many depend entirely on plastic products due to their useful material properties and lower production cost (Jain *et al*.,2015). To build our economy on a sustainable basis we need to find a replacement for fossil carbon as chemical industry feed stocks (Pei *et al*.,2011). For efficient management of used plastic materials, recycling is one solution, another solution to reduce plastic residue is the use of biodegradable plastics (Anish *et al*.,*2013*) Biomaterials are natural products that are synthesized and catabolized by different organisms and have found broad biotechnology application. They can be assimilate by many species (biodegradable) and do not cause toxic effects in the host, Like Gram negative bacteria, Gram Positive bacteria, Archaea, Cynobacteria,etc. Bio plastics are special type of biomaterial (Kumaravel *et al.,2010)*. They are polyesters produce by range of microbes, cultured under different conditions (Luengo *et al.,*2003). This polymer are usually lipid in nature are accumulated as a storage materials in form of mobile, amorphous, liquid granules allowing microbial survival in stress condition(Luengo *et al*.,2003).. Microbes belonging to more than 90 genera-inducing aerobes, anaerobes, photosynthetic bacteria, archaebacterial and lower eukaryotes are able to accumulate and catabolize there polyesters. The most widely produced microbial bioplastics are PHB, PHA and their derivatives(Kumaravel *et al.,2010)*.Bioplastics began to be recognized as a positive and important invention of the chemical and plastics industry and started proving various and numerous opportunities. PHAs, a biologically produced biodegradable substance which has similar characteristics of plastic have become a main focus for the research in finding substituent for plastic. (Kumaravel *et al.,2010)*PHB is water insoluble and relatively resistance to hydrolytic degradation, shows good oxygen permeability, good U-V resistance, but poor resistance to acids and basis, soluble in chloroform and suitable for medical application. Has melting point 175 and its nontoxic. Lemoigne first describe a bioplastic poly(3-hydroxy butyrate (PHB) in *Bacillus magisterium*. This initial remark was almost forgotten until the mid-1970s when because of petroleum crisis, a scientific movement aimed to learning alternative source of fossil fuel reserves was undertaken(Kumaravel *et al.,2010)* (Luengo *et al.,*2003).. In spites of this interesting properties, industrial production oh PHAs is still not well established. In the 1950s , North American company W.R.Grace Co. made the first attempt to produce PHB at commercial level.(Bhuwal *et al.,2013)* Bioplastic can produce from standard carbon sources (Jain etal.,2015).. Sugars have been shown to be an effective feed stock for PHA production in Brazil.(Kunasundari *et al.,2011)*. Renewable carbon sources such as sucrose, cellulose, and triacylglycerol. Extensive studies have been conducted on the use of inexpensive substrate ,including starch , glycerol, soybean oil, sugarcane bagasse, molasses and activated sludge (Florace *et al.*,2017).Also starch waste water , beer malt, soya waste, hydrolyzed whey permeate , glycerol water , olive oil mill waste water, waste frying oil , food scarps , hemicellulose , and corn sleep liquor were used for bioplastic production(Javers *etal.,2012)*. Numerous publications demonstrated that innumerable marine microbial species have intracellular poly - B – hydroxybutyrate particles as carbon and energy reserves inside their cells under serve stress condition.(Wala *et al.,2017)*This study aimed to screen the ability of bacteria that isolate from soil samples from different locations to produce bioplastic and is characterization and production of PHB using different raw wastes materials like potato peels, Molasses, Waste cooking oil and Orange peels.

I. Materials and methods.

1] Isolation of Polyhydroxybutyrate producing bacteria:-

For isolation of PHB producing bacteria different types of soil samples were collected from different part of South Gujarat region. Two different soils samples were collected.1)Dumas beach soil sample.2)Kholwad Garden soil sample.
The samples were stored at 4^0C until analysis. In 9 ml of sterile distilled water, 1gm of soil samples were dissolved, and spread on to nutrient agar plates with 2% glucose. 1ml of each dilution was spread on carbon rich nutrient agar plates.

2. Screening of PHB producing Bacteria

2.1Carbol fuchsin Staining:-

Carbol fuchsin staining is performed to determine the intracellular production of PHB by the isolates. A thin smear of all the isolates were stained with carbol fuchsin stain for 45 seconds. The isolates capable of producing PHB showed dark colored granules PHB intracellularly. (Kumari *et al*, 2013)

2.2Sudan Black B staining:-

PHB producing bacteria were further conformed using Sudan Black B staining method. Sudan Black B stain was prepared as 0.3% solution (w/v) in 60% ethanol. The smear of cultures was prepared on glass slides and heat fixed. The samples were stained for 10min with Sudan Black solution, rinsed with water and counter stained with 0.5% safranin for 5min. and observed at 100x magnification.(Bhat , *et al* 2017)

2.3Nile Blue A staining:-

Sudan Black B. positive isolates were checked for PHB production by Nile Blue A. staining, a more specific stain for polyhydroxybutyrate (PHB) by a more rapid and sensitive, viable colony method. This dye at concentrations of only 0.5µg/ml was added in 1ml of Dimethylsulphoxide (DMSO). After dissolving the dye it was added in carbon rich nutrient agar medium (glucose 1%, beef extract 0.3%, peptone 0.5%, sodium chloride 0.8%, and agar 1.5%) and growth of the cells occurred in presence of dye. This allowed an estimation of PHB in viable colonies at any time during the growth experiment and a powerful discrimination between PHB-negative and PHB-positive strains. The PHB accumulating colonies, after Nile Blue A. staining, showed bright orange fluorescence on irradiation with UV light and their fluorescence intensity increased with increase in PHB content of the bacterial cells. The isolates which showed bright orange fluorescence on irradiation with UV light after Nile blue A staining were selected as PHB accumulators.(Bhagowati *et al*, 2013).

2.4 Morphological characterization of PHB producing bacteria:-

2.4.1 Culture characterization of PHB isolates:-

The culture characteristics such as size, shape, margin, elevation, surface, opacity and pigmentation were studied after growing the most potential isolates on nutrient agar plates.

2.4.2Gram staining

2.4.3Identification of PHB producers by Biochemical tests:-

Biochemical tests were conducted manually by using respective culture media according to standard microbiological procedures for PHB isolates.

2.5 Preparation of Potato and Orange peel powder:-

All the peels were placed in two different sterile zip lock bag. Peels were dried under sunlight for 2-3 days. Dried peels were converted in to powder form with help of mixture grinder. Powder was stored in an air tight container.

2.6 Production of PHB from different wastes:-

2.6.1 Inoculum medium:-

Carbon rich medium (5 gm peptic digest, 5 gm sodium chloride, 1 gm beef extract, 1 gm yeast extract, 2% glucose, 1000 ml distilled water) used as Inoculum medium for the growth of PHB producing bacteria. The bacteria were inoculated in medium and incubated at 37^0C for 24 hr.

2.6.2 Production medium of PHB :-

Mineral salt medium [(4 gmNA2HPO4, 1gm KH2PO4, 0.2 gm MgSO4.7H2O, 0.05 gm CaCl2.2H2O, 3 gm (NH4)2SO4, 0.2 NaCl,) Molasses waste 5 gm in 100 ml MSM, Waste oil 5gm in 100 ml MSM, Orange peel waste 5gm in 100 ml MSM, potato peel waste 5gm in 100 ml MSM, Mix waste 5 gm in 100 ml MSM] was used for production of PHB on that different wastes like molasses, crude oil, orange peel waste, potato peel waste, mix waste (potato, pineapple, orange, lemon wastes) used as a carbon source. 5ml of culture from inoculum medium was added in production medium in sterile condition. The production flasks were incubated in shaking condition at 200 RPM at 37^0C. After 3 days of incubation in shaker the extraction of PHB was performed.

2.7 Extraction of PHB by using dispersions of sodium hypochlorite and chloroform:-

10ml of culture was centrifuge for 10min, and supernatant was discarded. The pellet was suspended in 2.5 ml of 4% sodium hypochlorite for digestion and 2.5 ml of chloroform, incubate at 37C for 1hr. The suspension was centrifuged at 3000 rpm for 10min. The suspension divided in three phase, upper phase contains sodium hypochlorite, middle phase contains cell debris and bottom phase containing PHB in chloroform. Upper 2 phase were removed first with pipette, the middle phase removed by filtration from the chloroform phase. Finally, PHB was recovered from the chloroform phase by nonsolvent precipitation (methanol and water 7:3 vol/vol). The nonsolvent allowed to evaporate for dryness at 30C to obtain PHB powder. (Hahn *et al*, 1995)

2.8 UV–Vis spectrophotometer analysis of PHB

The extracted PHB was dissolved in chloroform and scanned in the range of 200–250 nm against chloroform blank and the spectrum was analyzed for a sharp peak at 240 nm (Getachew *et al*,2016).

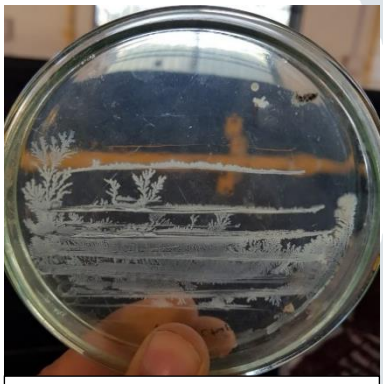
2.9 Calculation of PHB:-

This was calculated to determine the cellular weight and accumulation other than PHAs.with slight modification. The percentage of intracellular PHA accumulation is estimated as the percentage composition of PHA present in the dry cell weight:
Residual biomass (g/L) = DCW (g/L)−Dry weight of extracted PHB (g/L)
PHA accumulation (%) = Dry weight of extracted PHB (g/L) × 100%/DCW (g/L).(Bhuwal, *et al*, 2013)

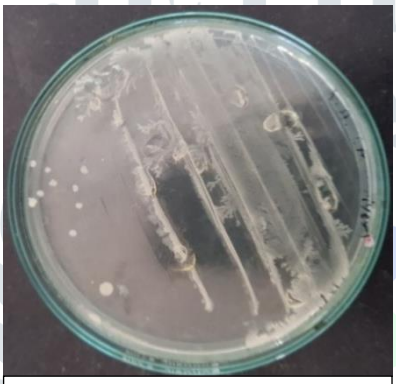
3 Results and discussion:-

3.1 Isolation of various bacteria:-

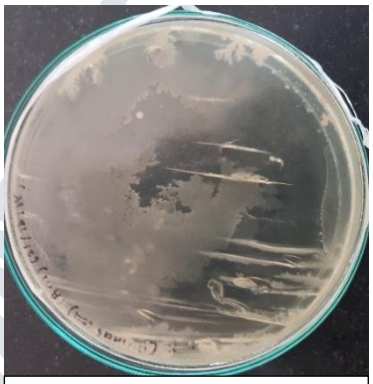
25 different types of bacteria were isolated from the soil samples. Figures show different types of bacteria isolated from soil samples. By sub-culturing pure cultures were obtained on nutrient agar plates.



Isolation of bacteria from soil sample



Isolation of bacteria from soil sample

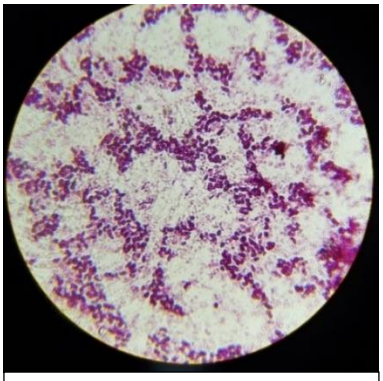


Isolation of bacteria from soil sample

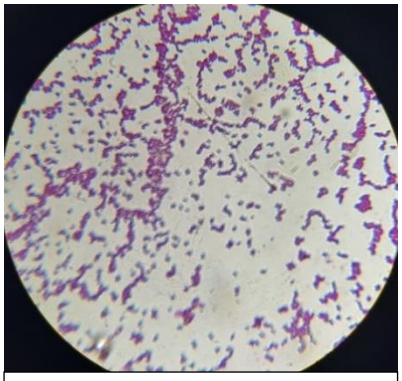
3.2 Screening of PHB producing bacteria:-

3.2.1 Carbol fuchsin Staining as a primary staining:-

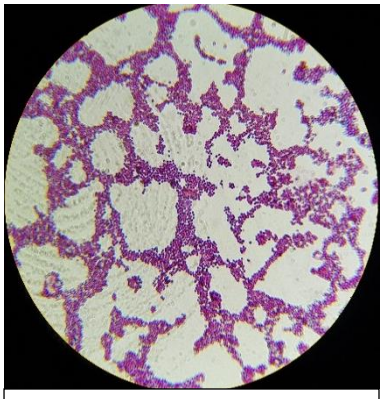
To distinguish PHB producer from non-producers, Carbol fuchsin staining was carried out. From 25 isolates, 10 isolates were found to have dark colored granules of PHB within their cells. Therefore it was assumed that the isolates were capable of producing PHB. For further conformation Sudan Black staining was carried out.



Carbol fuchsin staining of bacteria.



Carbol fuchsin staining of bacteria.



Carbol fuchsin staining of bacteria.

3.2.2 Sudan Black B. Staining:-

For confirmation of PHB granules, Sudan Black staining was carried out. Bacteria which showed dark granules in Carbol Fuchsin staining were selected for Sudan black staining. Among them 8 bacteria showed dark black to purple colored granules indicating positive for PHB granules. PHB producing granules were showed in figures,that confirm the presence of PHB .Same staining performed by various scientists on their research on PHB. For further conformation Nile blue A staining was performed.

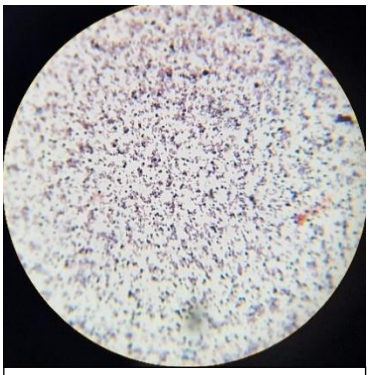


Fig:1 Isolation of bacteria from soil sample

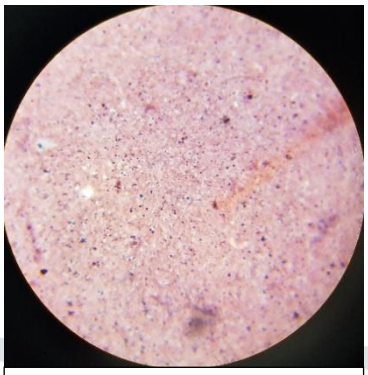


Fig:1 Isolation of bacteria from soil sample

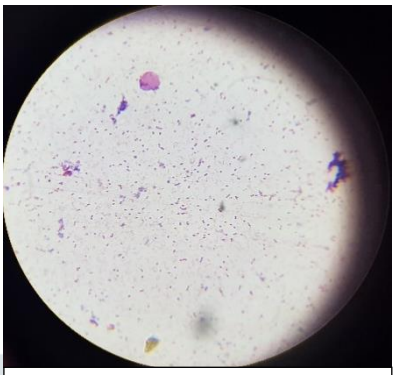
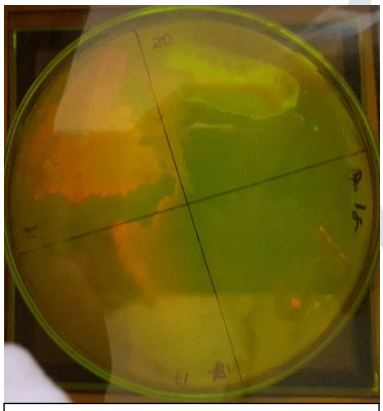


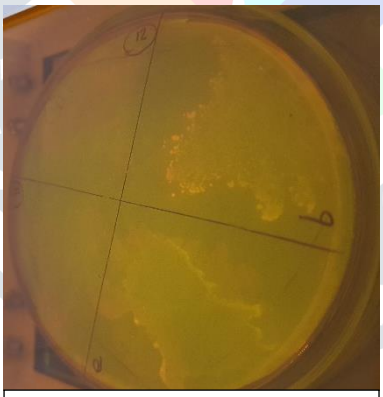
Fig:1 Isolation of bacteria from soil sample

3.2.3Nile Blue A. Staining:-

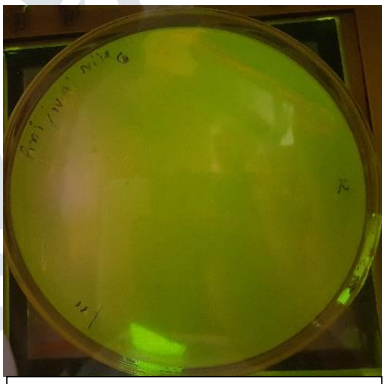
Nile Blue A. appeared to have a great affinity for PHB than the sudan black B. The selected isolates were streaked on media containing Nile Blue A. stain, the culture growth were placed under UV light for checking the fluorescence. 8 bacterial colonies gave orange fluorescence under UV-light indicating presence of PHB granules. Those colonies which showed more intensity in UV light, were selected for production of PHB. Figures below show positive result of Nile blue A. staining for PHB production.



Nile blue staining of isolated bacteria



Nile blue staining of isolated bacteria



Nile blue staining of isolated bacteria

3.3 Identification of PHB producing Bacteria:-

Those isolates which gave high fluorescence in UV light, were selected for PHB production. These Isolates were then identified by its morphological, colonial and biochemical characteristics.

Table 1- Characteristics of isolate (AA-3)

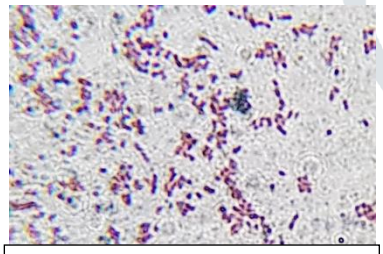
Colonies of isolate (AA-3)

characteristics of Isolate (AA-3):-	
Size	Small
Shape	Circular
Elevation	Flat
Edge	Entire
Color/Pigment	No pigmentation
Opacity	Translucent

Biochemical tests:-

A series of biochemical tests for identification of Isolate 3 (AA-3): Tests conducted include, 1- Glucose, 2- Sucrose, 3- Mannitol, 4-Xylose, 5-Maltose, 6-Urease, 7-M-R Test, 8-V-P Test, 9-Citrate, 10-Indole, 11-Catalase, 12-Nitrate reduction. Based upon biochemical tests results, the isolate were identified to be *Bacillus spp*. based on bergey's manual.

Table – 2 Gram's staining of isolate (AA-3)



Gram staining of isolate (AA-3)

Gram's Staining of Isolate:-	
Color	Violate
Shape of bacteria	Bacilli shape
Arrangement	Single and pair
Gram's behavior	Gram positive

Table – 3 Biochemical tests result of isolate (AA-3)

TESTS NAME	RESULTS
Glucose	Positive
Sucrose	Positive
Mannitol	Negative
Xylose	Negative
Maltose	Negative
Urea	Positive
M-R test	Negative
V-P test	Positive
Citrate	Positive
Indole	Negative
Catalase	Positive
Nitrate reduction	Positive

3.5 Production and extraction of PHB :-

Production of PHB using four different Carbon sources were carried out using *Bacillus spp.* The carbon sources used were potato peels, Orange peels, Molasses, and Waste cooking oil. The extraction of PHB from the fermentation broth was performed by sodium hypochlorite and chloroform method. During Extraction three layers were separated. There are many common techniques available for extraction of PHB, like solvent extraction methods(Hahn *et al*, 1995), Digestion methods,(Choi *et al*, 1999) Mechanical disruption(Harrison *et al*,1991), Supercritical fluid(Hejazi *et al*,2003).

Figures below showed the positive result of PHB.

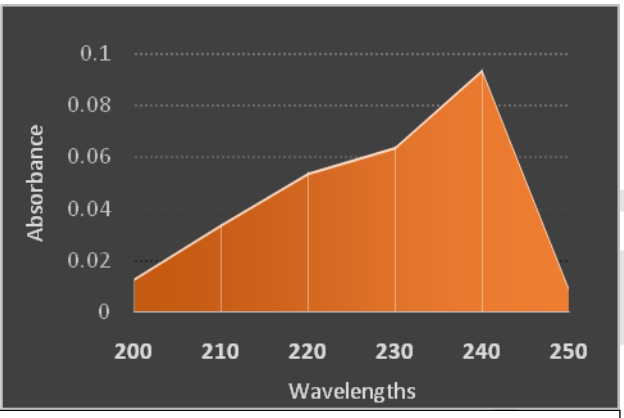
Separation of three layers

Separation of three layers

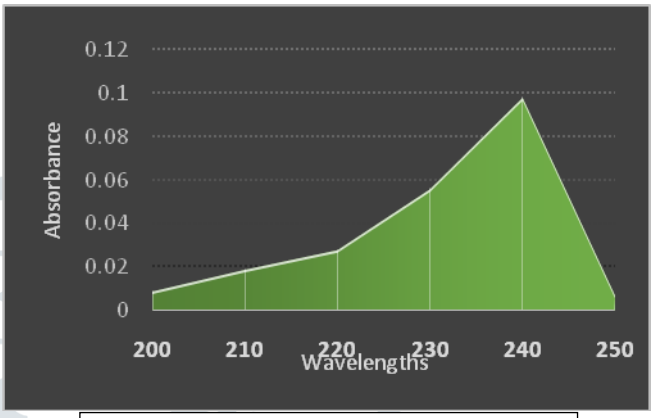
PHB present in chloroform

PHB present in chloroform

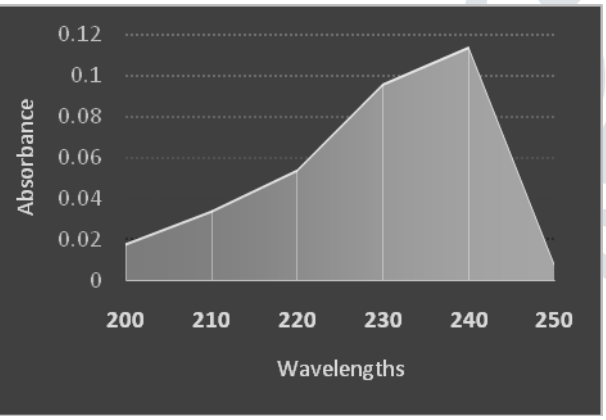
3.6 UV-VIS spectrophotometer for analysis of PHB.



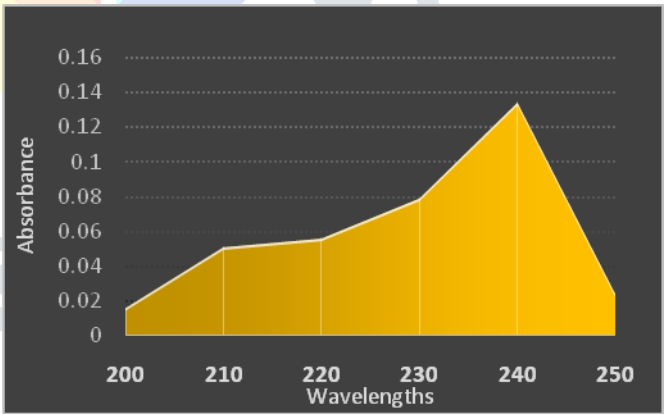
Scanning for PHB production in Potato peels waste



Scanning for PHB production in Molasses waste



Scanning for PHB production in Waste cooking oil

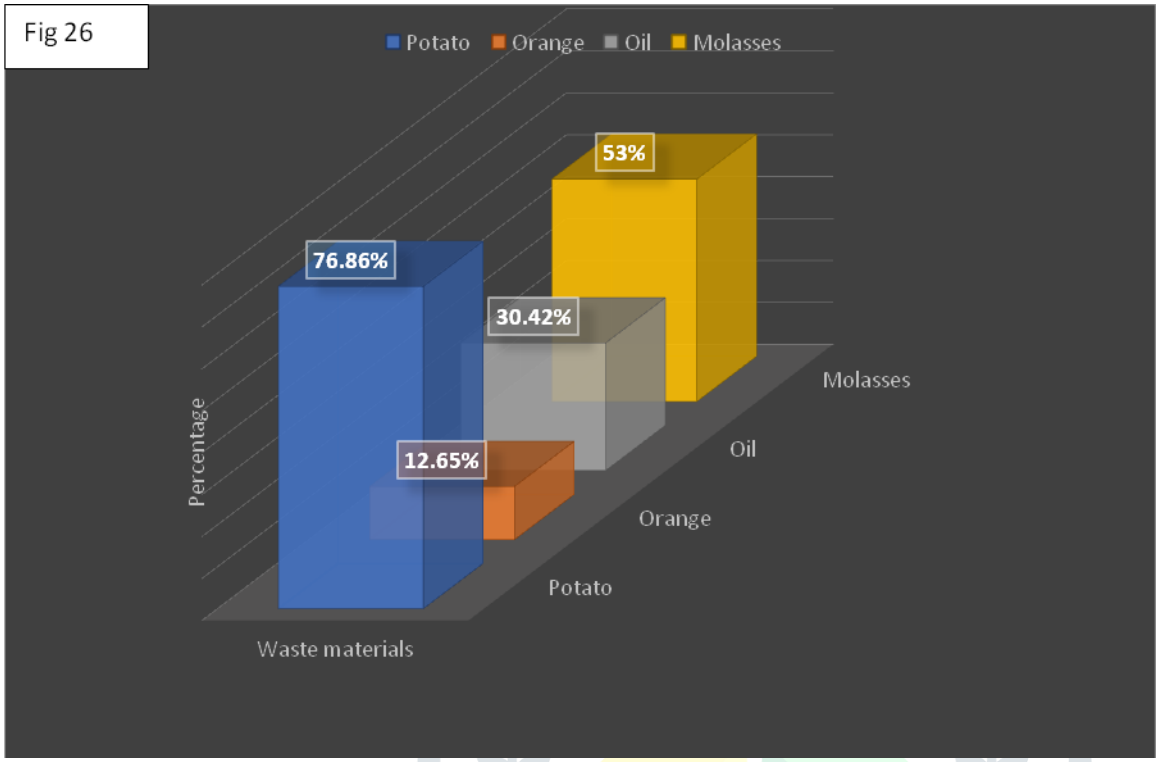


Scanning for PHB production in Orange peels waste

UV-Vis spectrophotometer scanning revealed that, the absorbance peaks were 240nm for Molasses, Potato peels, Orange peels, and cooking waste oil using *Bacillus spp.* Similar study was carried out by (Getachew *et al*,2016) using *Bacillus spp.*

3.7 PHB production from waste materials:-

Fig 26



PHB production was found to be maximum in Potato peel waste followed by Molasses, Waste cooking oil, and Orange peel waste using *Bacillus spp*..Similar studies of PHB production using *Bacillus spp*. Was previously done by Bhagowati (Bhagowati et al,2013), Leda et al,2009) (Wala et al, 2017).In contrast many scientists reported different organisms for production of PHB like P*seudomonas putida* KT217(Javers et al, 2012), *Pseudomonas oleovorans* (De Smet et al,1983), *Pseudomonas fluorescens* (Gamal , et al ,2013), *Cupriavidus necator* (Flores et al, 2017), Methanotrophic probacteria (Alliison et al, 2011)The highest 76.86% production was given from potato peels. After that from molasses 53% PHB was extracted in contrast Page et al (Page et al; 1992) using molasses as carbon source and extracted 66% of PHA using *A. vinelandii*. Nikodinovic et al ((Nikodinovic ,*et al* 2013) also used molasses as a carbon source for production of PHA.From waste cooking oil 30.42% PHB extracted. In contrast (Gamal 2012) produce 29.65% PHA using *pseudomonas sp.* Pagliano et al(Pagliano et al 2017) used waste cooking oil for production of PHB. In certain cases as a carbon sources avocado oil (Flores *et al*,2017), Whey lactose (Koller *et al.*,2012) were used for production.

Conclusion:-

In the 21st century we are living in a load of pollution from many causes including polythene wastes, hence the search for a suitable, economical, harmless alternative is of huge demand. Bioplastics are the most suitable for this cause. In this favor, the current study revealed the presence of many PHB producer bacteria in both the environments studied which can be used for production of bioplastics in both laboratory as well as industrial scale. The characterization of PHB by various analytical techniques showed the production of pure PHB by the selected isolates which can be studied further by various blending techniques to get a more user friendly, economical goods. The most potent among the isolates were identified to be Bacillus *spp*., which are ubiquitous in nature and have been reported to possess the capability of overcoming the stress conditions by countless mechanisms. Though Bacillus *spp*. have been reported to be PHB producers.

Bacillus spp. gave more production of PHB in Organic raw wastes like potato peels other than waste cooking oil, Orange peels waste, and Molasses. UV-Vis spectrometer conform the presence of PHB in production medium.

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