March-April 2020
ISSN: 0193-4120 Page No. 5468 - 5479

# Isolation and Partial Characterization of Biopolymer (PHB) Produced by Marine Bacterium Actinobacterium SP Associated with a Marine Gastropod Purpura Bufo

**N. Ezhilraj[1], S. Remya[2], S. Mary Josephine Punitha[3*]**

[1,2,3*]Centre for Marine Science and Technology, Marina Campus, Manonmaniam Sundaranar University, Rajakkamangalam

***Article Info***
***Volume 83***
***Page Number: 5468 - 5479***
***Publication Issue:***
***March - April 2020***

***Article History***
***Article Received****: 24 July 2019*
***Revised****: 12 September 2019*
***Accepted****: 15 February 2020*
***Publication****: 28 March 2020*

***Abstract:***
Twenty bacterial strains were isolated from the surface of a marine gastropod *Purpura bufo* and screened for biopolymer (PHB) production using Sudan black- B staining. Among the 20 bacterial strains, only three strains, which showed positive on PHB production were selected and named as GS4 GS10 and GS16. The PHB produced by the selected strains were individually quantified and their physico-chemical properties were characterized. The PHB was produced and they optimized for different medium, carbon nitrogen sources and changes of pH and temperature. The result revealed that a maximum production of PHB was found in the medium added with fructose (79.19%), potassium nitrate (85.93%), 20% NaCl (96.91), 30°C (98.17) and $p^H$ 6 (95.87). The biopolymers were TLC used to the compounds is identified (esters, alkyl bromide and aromatic).FTIR is the technique used to the functional groups is identified. The bacterium responsible for the production of biopolymer was identified as *Actinobacterium sp* through 16SrRNA gene sequencing. Further purification and characterization will lead to a novel natural based biopolymer from the marine bacterium to replace the chemically synthesized non-degradable polymers.

***Keywords****: Biopolymer; Marine bacteria; poly-β-hydroxybutyrate; Sudan Black -B.*

## Introduction

Biopolymer is an organic substance that is produced naturally by living species. This include plants of many types, such as corn and soya beans, can also come from different types of trees and even some bacteria.Proteins, nucleic acid, fats & polysaccharides are molecules which forms long chain to form polymers[1]. These polymers perform several important functions. They also contribute an essential parts in structural components of tissues, cells & the whole organism[2]. The biopolymers are generally varied in seven different classes named PHBs, polyisoprenes, polynucleotides, lignin, polyamides, polysacchrides and polyphosphate.

PHBs are type of biodegradable plastic which is bio compactable & have wide variety of physical properties same as polypropylene [3]. PHBs are produced in nature by several microorganisms such as yeast, fungi and mostly by various microorganisms such as Bacillus sp., Pseudomonas sp., Cupriavidus sp. and Aeromonas sp., are used in research to know their usage for production of high capacity for the production of PHB [4]. Mostly Researchers uses species named Bacillus megaterium, Ralstonia eutropha. B.megaterium can contribute around 84% for the PHB production. Apart from this several other organisms also have the capability to produce PHA from organic waste. Environments such as marine microbes, sewage,

municipal sludge etc have various microorganisms and has isolated PHBs from this environment [5]. Under stressed growth conditions such as lack of oxygen & nitrogen bacteria can accumulate PHBs as carbon & energy [6]. The bio-plastic production can be made by specific bacteria such as Alcaligene slatus, Azotobacter vinelandii, E. coli, Methylotrophs, Paracoccus denitrificans, Pseudomonas olevorans.Moreover, it has been reported that bacterial strains such as Haloferax sp., Microbacterium barkeri, Alcaligenes sp. and Bacillus sp. (Kulpreecha et al., 2009) can synthesize and accumulate PHBs in their cells [7].

The bio compactable & biodegradable bio polyesters that shows thermal property same as polypropylene (petroleum based polymer) and is bio based these are the properties of PHBs [8] . PHBs and its subordinates are used as bio plastics & have several medical applications mainly in delivery of drugs in engineering tissues, & packing of food. Because of biodegradable nature, use of biopolymers are highly appreciated as it avoids the use of oil based polymers which is a recalcitrant and has the ability to accumulate in the environment [9]. Even through variety of microbial bio polymers are available in the environment, only few are marketed [10]. For this marketing screening of bacteria that produce different kinds of bio polymers are done and the usage of substrate is needed for production of biopolymer [11]. Considering above facts, the research was undertaken to isolate and characterize the PHB producing Actinobacterium sp associated with a marine gastropod Purpura bufo [12,13,14].

## 2. Material & Methods

### 2.1. Isolation & screening of bacteria that produce biopolymer

Marine gastropod *Purpura bufo,* were collected from the rocky shore region of Colachel coast that is located in the West coast of India. The collected gastropods are taken to labs aseptically by keeping them in seawater in a plastic container. In the labs, the collected samples were washed gently with sterile sea water for removal of the organisms that are loosely attached and the bacteria present in the surface were swabbed using sterile cotton buds & is mixed with in 1 ml of sterilized & filtered seawater. The bacterial suspension was diluted serially to various concentrations and placed in agar plates & incubated at 37$^{o}$C for 24 h to get pure bacterial colonies. And has isolated 20 bacterial isolates that are morphologically distinct and all these isolates of bacteria were screened to ensure the production of PHB followed by Sudan Black-B dye method. For massive screening of PHB producers, NA medium supplemented with 1% glucose was sterilized by autoclaving at 121$^{o}$C for twenty minutes and brought down to room temperature. Then the medium was streamed into sterile petri plates & is settled in order to solidify. Each plate was marked & differentiated in to 4 equal parts and an individual bacterial isolate was spotted in each part. Then the plates were incubated at thirty degree Celsius twenty four hours. Sudan black B dye with 0.02% ethanol are poured over the colonies of bacteria in the plate & is kept for thirty minutes undisturbed. Later the plates were washed with 96% ethanol to remove the excess stain. The colonies that showed up dark blue colonies are positive for producing PHB [15,16,17].

#### 2.2.1. Quantification of poly - hydroxy butyrate (PHB)

In the initial screening study, among the tested 20 bacterial isolates, only 3 isolates displayed PHB production. Then these three positive isolates of Sudan black B were taken for studying measurement of production of PHB. Positive strains of PHB were grown individually in zobell marine broth. Then the broths with bio polymer were centrifuged individually at 10,000 rpm for 10 minutes & pellets were cleaned with acetone & ethanol for removal of unwanted materials. Followed by pellets were suspended individually in 4% of NAOCl of equal volume & is incubated for thirty minutes in room temperature. Again the whole mixture was centrifuged & the supernatant were discarded. The

*March-April 2020*
*ISSN: 0193-4120 Page No. 5468 - 5479*

individual pellets of cells containing PHB were again washed with $C_3H_6O$ & $C_2H_5OH$. The granules of polymer were mixed with hot chloroform and is evaporated & PHB was produced & was weighed & noted [18,19,20].

The granules of the polymers were dissolved in Conc. $H_2SO_4$ (1mg/ml) & was heated at 100ºC for ten minutes for conversion of PHB to crotonic acid (brown colour). The heated solution was cooled down and at 260nm the absorbance was taken against Conc. $H_2SO_4$ kept as blank in the spectrophotometer. A standards diagram was prepared with pure PHB (Sigma, Aldrich) with conc. 20-100µg/mL (Law and Slepecky, 1969). The amount of PHB produced are displayed by comparing it with the standard. Based on the PHB yield, only one promising bacterial isolate, which produced maximum amount of PHB was selected for further studies.

**2.3. Antibiotic sensitivity Test**

To test the antibiotic sensitivity nature of the promising isolate, 100µl of culture was swabbed on zobell marine agar medium. Then 5 different types of antibiotic discs Tetracyclin $TE^{30}$, Amphotericin – B (AP), Flucanazole $FLC^{25}$, Streptomycin $S^{10}$, Itraconazol $IT^{10}$ kept in medium & were incubated for a day at 37°C [21,22].

**2.4. Culture optimization for production of PHB**

**2.4.1. Impact of Carbon on production of PHB**

In basal medium ( Zobell Marine Broth : ZMB), 1% each of variety carbon sources named as glucose, sucrose, fructose, lactose & galactose were mixed. To this selected bacterial strains were inoculated & incubated at 37ºC for 3 days, in order to study the impact of various carbon sources on PHB production. After incubation, production of PHB was determined as above method [23,24].

**2.4.2. Impact of nitrogen sources on production of PHB**

To assess impact of several sources of nitrogen on production of PHB, the various $N_2$ sources like $(NH4)_2SO_4$, $NH_4Cl$, sodium nitrate, urea & potassium nitrate were used. For this, basal culture media was supplemented with 1% each of individual nitrogen sources, then inoculated with the selected bacterial strain and incubated the setup for 3 days at 37ºC. Later the production of PHB production was determined [25,26].

**2.4.3. Impact of Sodium chloride on production of PHB**

In order to assess the impact of sodium chloride on production of PHB by the promising bacterial strain, the culture was grown in the basal medium amended with different concentrations of NaCl (1, 5, 10, 15 and 20%) and incubated the setup at 37ºC for 72 h. . Later the production of PHB production was determined [27].

**2.4.4. Impact of temperature on production of PHB**

The picked bacterial strain was inoculated in the basal culture media and incubated at different temperature *viz* 25,30,35,40 and 45ºC for optimization. After 3 days of incubation, production of PHB was determined [28,29.30]].

**2.4.5. Impact of pH on production of PHB**

The selected strain of bacteria was inoculated to the basal medium prepared with various pH (pH 5,6,7,8 and 9) and incubated at 37ºC for 72 h to learn the impact of pH variation on PHB production [31,32].

**2.5. Determination of physico-chemical properties of PHB**

**2.5.1. Solubility test**

In order to find out the solubility of PHB, 100mg each of PHB pellet was taken in different vials, to this 2ml of different solvents such as water, acetone, chloroform, ethanol, butanol and benzene were added individually, further mixed thoroughly

and observed for the solubility of PHB in the respective solutions [33,34].

### 2.5.2. Chemical property

The standard procedure proposed by Lowery *et al.* (1951) was followed to estimate the protein content in the PHB pellet. Lipid content in the PHB was estimated through the procedure of Folch *et al.* (1957). Similarly carbohydrate content in PHB was estimated by the standard procedure of Dubois *et al.* (1956) [35,36].

### 2.6. Partial Purification of PHB through TLC

Thin layer chromatography (TLC) technique was followed for the partial purification of PHB produced by the potent bacterium. The biopolymer that is extracted was poured over the glass plates that are activated by silica gel, later the activated plates were kept for TLC in Thin layer chromatography chamber with mobile phase with $CH_3OH$, acetic acid & benzene in the ratio 3:1:1. Once the solvent reach the end point the plates were taken off the chamber & kept in other chamber with crystals of Iodine to help in the compound development. The spots with the compound are scrapped off the layer & is dissolved with respective solvents & is centrifuged for fifteen minutes for separating the supernatant that is the purified biopolymer [37,38].

### 2.7. Fourier Transform Infra-Red analysis (FTIR)

SHIMADZU FT-IR system the spectra of FT- IR was noted. Less quantity of purified biopolymer by TLC placed on surface of heavily polished plate of KBr salt & another plate of KBr was kept over the plate to spread the compound of thin layer [39].

### 2.8. Recongnisation of potent PHB producing strain using 16S rRNA gene sequencing

The DNA was isolated from the pure culture of the potent PHB producing bacterium and the 1.4kb rDNA fragment was multiplied using high-fedelity PCR. By using primers named 16s (5'AGAGTRTGATCMTYGCTWAC-3') & 16s (5'-CGYTAMCTTWTTACGRCT-3') the 16s rDNA was multiplied with PCR. The size of the product was 1.5kb & the same primers were used for its sequencing & for confirmation of the sequence other internal primer was used. The nucleotide of the test strain that is obtained after 16S rRNA sequence were undergone with BLAST analysis & the Phylogenetic tree was obtained from other database of NCBI [40].

## 3. Results

### 3.1. Selection of Bacterial isolates for PHB production and quantification

Sudan Black B- dye staining method used to the 20 different bacterial species are isolates and initially screened for PHP production. Among the tested 20 isolates, only 3 isolates (GS4, GS10 and GS16) gave the positive result on PHB accumulation. Finally three different isolates were quantitative estimation for PHB production. It was noticed that, the production of PHB by the 3 strain such as 0.583, 0.354 and 0.231g/100ml these al concentrations bacterial was growth in the log phase.Among the three bacterial isolates, the maximum PHB yielding bacterial strain (GS4) was selected for further studies ( Table 1).

Table 1: PHB Production by the potent *actinobacterium* strain

| S.NO | STRAINS | PHB(%) |
|---|---|---|
| 1 | GS4 | 57.15 |
| 2 | GS10 | 25.89 |
| 3 | GS16 | 30.70 |

March-April 2020
ISSN: 0193-4120 Page No. 5468 - 5479

### 3.2 Optimization of culture medium for PHB production using various parameters

Optimization of the PHB maximum produced with different nutrient sources like carbon, nitrogen and Nacl concentrations and other parameters like pH and temperature. The results obtained are noted in table 2

Table 2: List of antibiotics used to measure the sensitivity along with zone diameter

| S. No | Antibiotics | GS | Diameter of Zone of inhibition(mm) |
|---|---|---|---|
| 1 | Tetracyclin | Sensitive | 3 |
| 2 | Amphotericin - B | Resistant | Nil |
| 3 | Flucanazole | Resistant | Nil |
| 4 | Streptomycin | Sensitive | 2.5 |
| 5 | Itraconazol | Resistant | Nil |

### 3.3 Effect of carbon source on PHB production

The PHB production was analyzed in the different effect of carbon sources in medium amended with five different carbon sources such as glucose, sucrose, fructose, lactose and galactose. The result showed a marginal variation on PHB production with different carbon sources and the maximum yield was observed in the medium supplemented with 1% fructose. It was significantly higher (79.19%), when compared to other tested carbon sources like lactose (64.74%), glucose (64.93%) and galactose (60.62%), however the lowest (48.04%) yield of PHB was recorded in the medium substituted with sucrose (Fig .1).

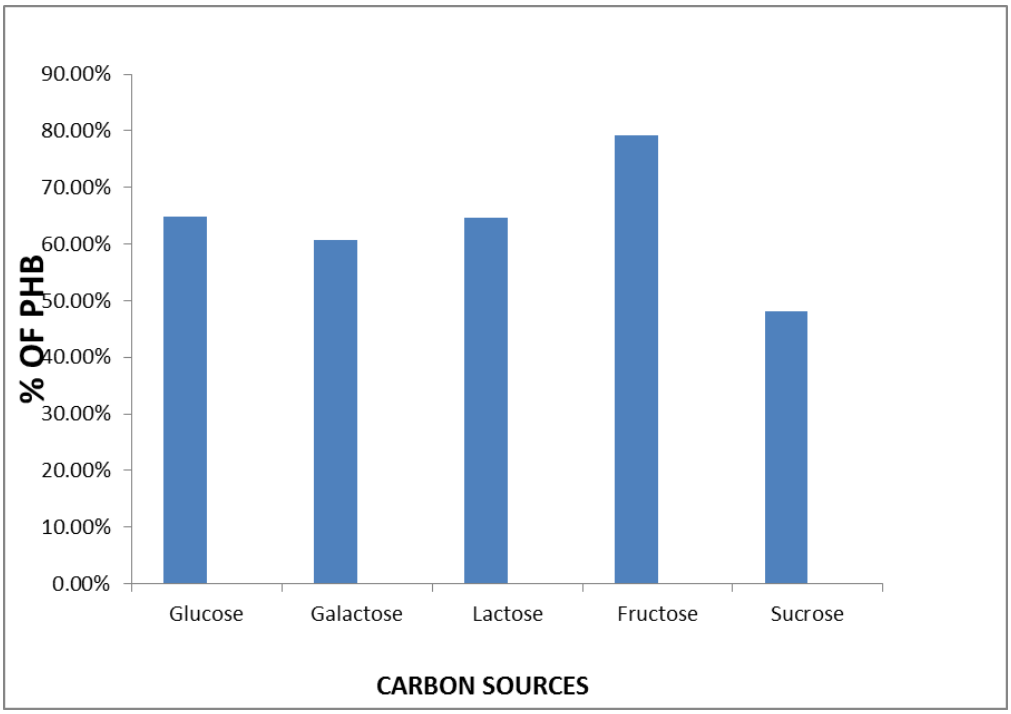



Fig 1: Effect of Different Carbon Sources On PHB Production

### 3.4 Effect of Nitrogen source on PHB production

The PHB production of different stains are tested with the five nitrogen sources. Among the tested five nitrogen sources, potassium nitrate influenced more on PHB production when compared to other tested nitrogen sources with the maximum PHB production of 85.93%. Followed by urea (69.25%)

March-April 2020
ISSN: 0193-4120 Page No. 5468 - 5479

and ammonium sulphate (60.52%). The PHB production was finally recorded in the nitrogen source of as ammonium nitrate and ammonium chloride was recorded as 50.76 and 29.51% respectively (Fig. 2).

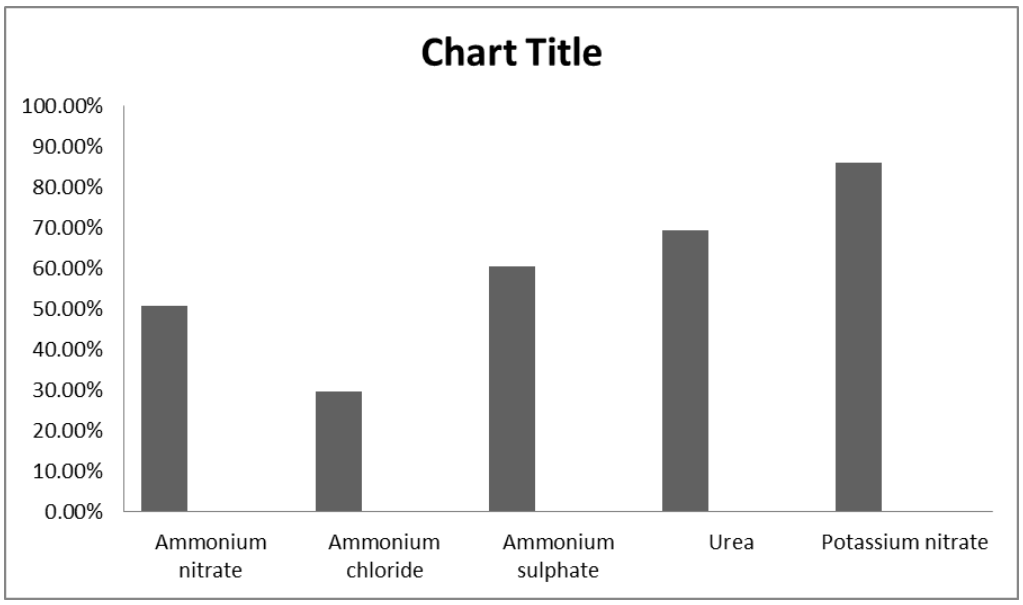



Fig 2: Effect of Different Nitrogen Sources On PHB Production

### 3.5 Effect of different concentration NaCl on PHB production

PHB production was observed in the medium substituted with various concentrations of NaCl (1, 5, 10, 15 and 20%) and it is observed that maximum (96.91%) PHB production was recorded at 20% of NaCl concentration. They compared with Nacl concentration the maximum PHB was produced.The next suitable concentration was found to be 15% with the PHB yield of 93.66%. The percentage of PHB yield of 80.79% and 66.31% were observed for 10 and 5% of NaCl respectively. The lowest yield 41.50% was recorded in the medium supplemented with 1% of NaCl concentration (Fig. 3).

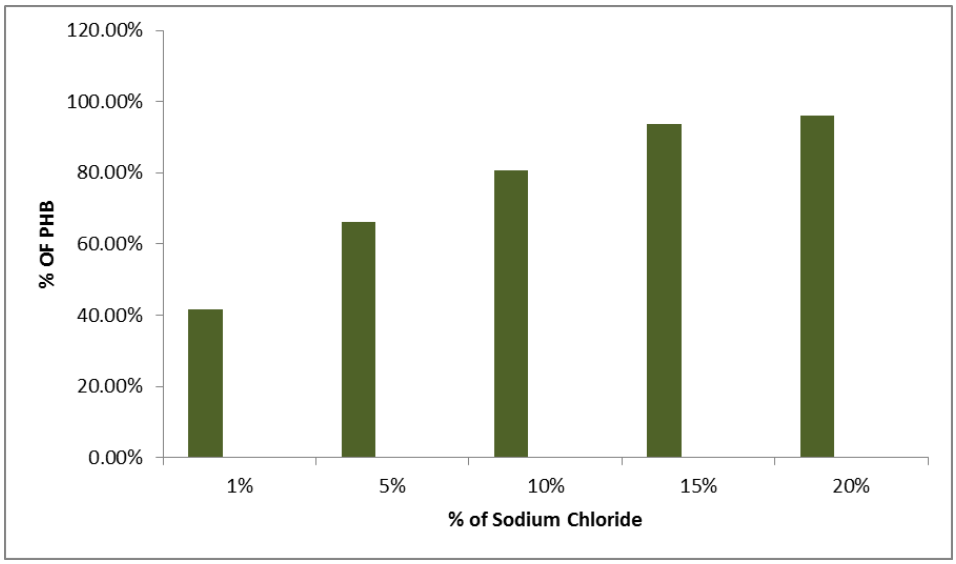



Fig 3: Effect of Different Nacl Concentration sources On PHB Production

March-April 2020
ISSN: 0193-4120 Page No. 5468 - 5479

### 3.6 Effect of temperature on PHB production

The optimal growth of temperatures is used to produce PHB. The five different temperature (25, 30, 35, 40 and 45°C) was used to the basal culture medium was incubated. The maximum production of PHB was observed at 30°C and it was found to be the optimum temperature with the yield of 98.17% PHB, followed by 25, 35 and 40°C with the yield of 96.55, 95.11 and 94.60% respectively. The lowest yield of PHB production was observed at 45°C with the percentage yield of 92.73% (Fig. 4).

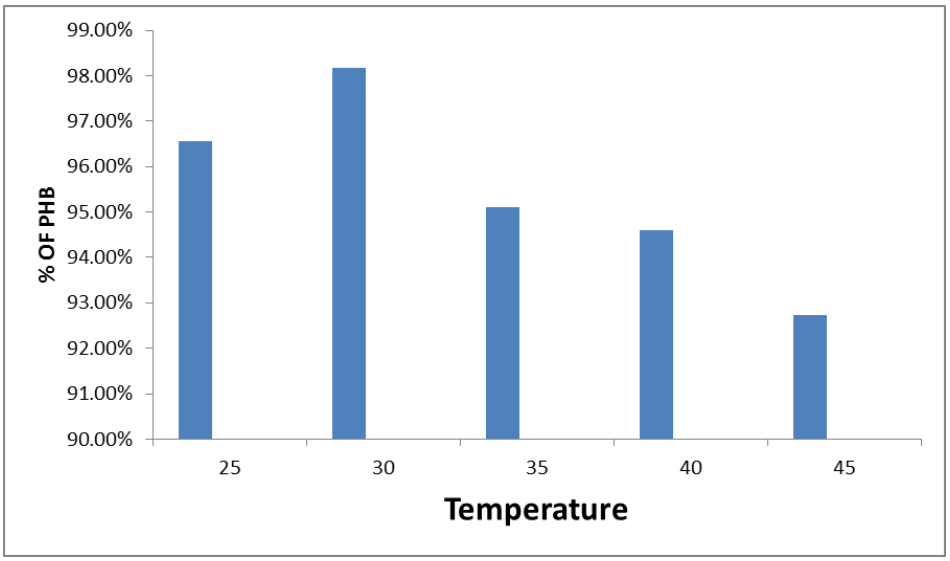



Fig 4: Effect of different medium temperature on PHB production

### 3.7 Effect of pH on PHB production

The different pH level was changes in the medium and the PHB production was analyzed such as pH 5,6,7,8 and 9. Among these, pH6 was found to be optimum with the PHB yield of 95.87% followed by pH 5 (94.76%). The PHB yield of 80.93% was recorded for pH 7. At pH 7 and 8, minimum level of PHB yield (61.30% and 61.25%) was recorded (Fig .5).

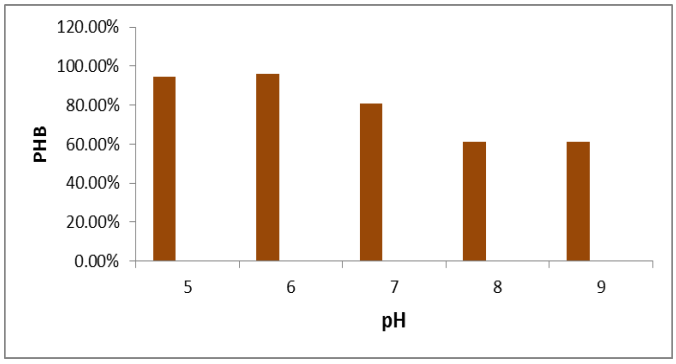
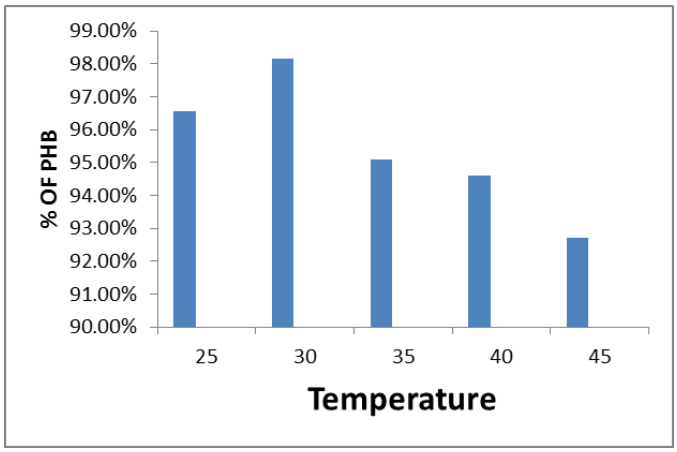



Fig 5: Effect of different media p$^{H}$ sources on PHB production




### 3.8. Characterization of PHB

The PHB extracted from the optimized medium was readily soluble in benzene, butanol, and acetone and partially soluble in chloroform but it was insoluble in ethanol and water. The chemical properties of the extracted PHB such as protein, lipid and carbohydrates were estimated to be 42.15µg/ml, 4.01µg/ml and 10.96µg/ml respectively (Tab 3)

March-April 2020
ISSN: 0193-4120 Page No. 5468 - 5479

Table 3: Chemical properties of PHB producing bacteria

| S.NO | STRAIN | CHEMICAL PROPERTIES | | |
|---|---|---|---|---|
| 1 | GS | PROTEIN (µg/ml) | CARBOHYDRATES (µg/ml) | LIPID (µg/ml) |
| | | 42.15 | 10.96 | 4.01 |

Fig 6: Thin Layer Chromatogram Of EPS Isolated From The Bacterial Strain (The Separated Spot Has Been Marked With An *)

### 3.9. Thin layer chromatography analysis of EPS

The active compounds are identified in silica gel glass plate method used EPS pellet are obtained and loaded. TLC was used to the compound position and Rf values are calculated 0.88cm (Fig 6).

### 3.10. Fourier Transform Infrared (FT-IR) analysis

FTIR spectrum use to the functional groups is identified in the EPS stain of GS. The groups are such as esters, alkyl bromide and aromatic compound. C-O stretches in between 1300-1000 $cm^{-1}$ indicated the presence of ester. Likewise, a narrow C-H stretch at near 670 $cm^{-1}$ indicated the presence of alkyl bromide. Besides, a C-H bending between 900-690 $cm^{-1}$ indicated the presence of aromatic compound (Fig .7).

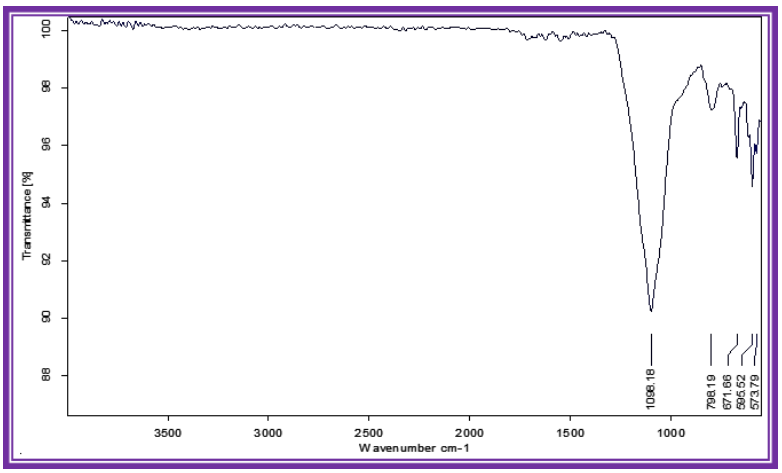



Fig 7: FT-IR spectrum of EPS of strain GS

### 3.11. Molecular identification and Evolutionary analysis of potent strain

BLAST is a sequence similarity program used to these sequences are compared with rRNA sequences. The computer based program method was used to the genes are compared. The evolutionary analysis

March-April 2020
ISSN: 0193-4120 Page No. 5468 - 5479

of *Actinobacterium* sp 99% homology was identified in the isolate GS. (Fig.8).

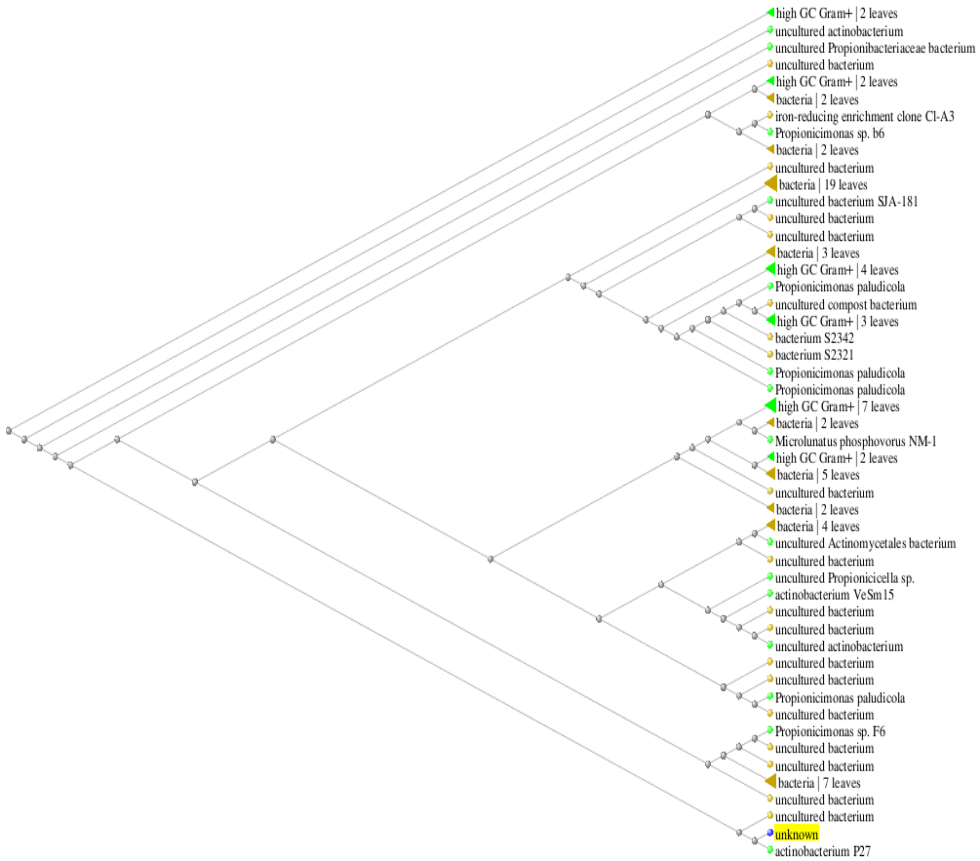



Fig 8: 16s rRNA sequence of the best strain GS isolated from the surface of gastropod

## 4. Discussion

Marine bacteria contain structure and biological activity. Biodegradable thermo polyesters are synthesized in the bacteria in under stress condition. Sudan Black- B staining method used to the biopolymers is synthesized in the *Purpura bufo.* Sudan Black- B dye staining used to the PHB is produced. These same method used to PHA are producing in bacterial stain form soil. Comparative analysis of the 20 bacterial stains the best three results are observed and positive stains are used for the PHB production. Also fifty soil samples bacteria also indicate positive results the black color granules are indicates PHB production.

The physical property of the produced PHB was tested for its solubility nature and it become discovered that the PHB produced by way of the pressure GS changed into soluble in but anol, acetone and benzene, however insoluble in water, ethanol and partly soluble in chloroform. Likewise, Uhlinger and White (1983) analysed the solubility of a biopolymer and determined that it changed into insoluble in distilled water and ethanol. The extracellular polysaccharids polymer from marine Pseudomonas remoted from marine sediments. Similarly, the chemical properties have been additionally analysed by means of estimating protein, carbohydrate and lipids, content material of the produced PHB. The result discovered that the PHB isolated from the pressure GS4 has forty two.15µg/ml protein 10.Ninety six µg/ml carbohydrate and four.01µg/ml lipid contents. In accordance with those Hoagland et al., 1993 pronounced that EPS of diatoms incorporate truthful quantity of protein, carbohydrates, lipids. However, in gift examine, best fewer quantities of carbohydrate and lipids were envisioned.

It has been stated that it's far possible to increase the polymer manufacturing by means of

manipulating the way of life situations [41]. Optimization of fermentation situations has been used to decorate the manufacturing of biopolymer. Hence, inside the present look at in order to maximize PHB manufacturing, diverse bodily and chemical factors along with carbon supply, nitrogen supply, pH and temperature had been optimized. The end result confirmed that the medium brought with fructose gave greater biopolymer production than different sources (Nitrogen, temperature, Nacl and pH). It has been reported that microorganisms are known to provide extra polymers when grown in carbon rich medium [42]. Borah et al., 2002 suggested using sucrose as the less expensive supply for the production of PHB. In the present observe, a few of the examined five one of a kind carbon sources (glucose, galactose, lactose, fructose and sucrose) most (79.19%) PHB manufacturing become found in the medium supplemented with 1% of fructose.

Similarly, 5 different nitrogen resources (ammonium nitrate, ammonium chloride, ammonium sulphate, urea and potassium nitrate) had been supplemented with basal medium. Among them 1% of potassium nitrate supported maximum (85.93%) PHB production and it became the most suitable source of nitrogen. Supporting, in a observe by using Aslim et al., 2002 said that potassium nitrate is the high-quality nitrogen supply for the production of biopolymer. In assessment, the have a look at by Yuksekdag et al., 2004 recommended that the best level of PHB accumulation turned into located within the medium supplemented with ammonium sulphate. Also Beyatliand et al., 2004 portrayed that most biopolymer production was received when the medium become supplemented with 20% sodium chloride.

The physical parameters like pH and temperature additionally play vital position in the production of PHB. In the present study bacterial strain GS turned into grown inside the basal medium amended with different pH degree (5, 6, 7, 8 and nine) and temperatures. The results depicted that maximum PHB manufacturing become accomplished at pH6 (ninety five.87%). Similarly, many of the examined temperatures, maximum PHB production became determined on the temperature of 30oC (98.17%). Similar end result changed into located by means of Bonartseva et al., 1994 who mentioned that Rhizobium sp. Produced excessive quantity of PHB at 30oC. On the other hand, Pozo et al., 2002 reported that pH 8 and temperature 500C have been observed to be optimum to get more PHB in Rhizobium sp.

Thin layer chromatography is a powerful technique that is being used for the separation and purification of natural merchandise. There are reviews about the purification of exopolymers the usage of TLC [43]. In the prevailing study the PHB turned into in part purified through the usage of TLC which gave only one compound with RF price of zero.88cm.Besides, the useful corporations gift inside the purified PHB had been characterised the use of FT-IR. The peaks appeared inside the FT-IR spectrum, which may additionally incorporate the purposeful corporations including esters, alkyl bromide and fragrant compound. In accordance with those Viju et al., 2014 mentioned the presence of functional agencies together with alcohol, alkenes, amines, esters and carboxylic acid corporations inside the exopolymer produced through the marine bacterium Pseudomons taiwansis which changed into identified the use of 16S rRNA gene sequencing analysis. In the equal way, in the gift examine, the bacterium liable for the production of biopolymer became identified as Actinobacterium sp. Via 16s rRNA gene sequencing. Similarly, Tanamool et al. (2013) isolated a biopolymer generating bacterium from soil in sugarcane plantation location and recognized as Hydrogenophaga the use of 16S rRNA analysis.Nallusamy et al. (2013) recognized five traces that produced PHB have been Pantoe agglomerans (Ca3), Bacillus subtilis (Ca4), Enterobacter sp. (L2) and Pseudomonas sp. (L2 &L4).Based on the findings of the study, it is concluded that the bacterium Actinobacterium sp. Can be used as a supply for the production of biopolymer. Further studies related to

characterization might cause the isolation of novel biodegradable polymer and which may be utilized in area of non-degradable polymers[44,45].

REFERENCES

1. Abu GO, Weiner RM, Rice J and Colwell RR (1991)Properties of an extracellular adhesive polymer from the marine bacterium Shewanellacolwelliana.Biofouling 3: 69–84.
2. Anderson AJ, and Dawes EA (1990).Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates.Microbiol.Rev.54: 450-472.
3. Arun A, Arthi R, Shanmugabalaji V and Eyini M (2009).Bioresour. Technol. 100: 2320-2323.
4. Aslim B, Yuksekdag ZN and Beyatli Y (2002). Determination of PHB growth quantities of certain Bacillus species isolated from soil. YurkishElctronic J. Biotechnol., 17: 24-30.
5. Atlic M, Koller D, Scherzer C, Kutschera E, Grillo-Fernandes P and Horvat(2011). Continuous production of poly- ([R]-3-hydroxybutyrate) by Cupriavidusnecator in a multistage bioreactor cascade, Appl. Microbiol. Biotechnol91: 295–304.
6. Bonartseva GA, Myshkina VL and Zagreba ED (1994).Poly-β-hydroxybutrate content in cells of various Rhizobium species during growth with different carbon and nitrogen source., Microbiol.,63:45-48.
7. Borah B, Thakur PS and Nigam JN (2002).The influence of nutritional and environmental conditions on the accumulation of poly-hydroxybutyrate in Bacillus mycoides RLJB-017.J.Appl.microbiol. 92:776-783.
8. Choi J, and Lee SY (1999).Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation, Appl.Microbiol. Biotechnol.51:13–21.
9. De Souza AM and Sutherland IW (1990).Exopolysaccharide and storage polymer production in Enterobacteraerogenes type 8 strains.Journal of Applied Bacteriology.76: 463-468.
10. Dubois M, Giles KA, Hamilton JK, Rebors PA and Smith F (1956).Caloriemetric method for determination of sugars and related substances. Anal Chem; 28: 350-356.
11. Folch J, LeesM and Stanley (1957).A simple method for the isolation and purification of total lipids from animal tissues.J.Biol. chem.., 226: 497-509
12. Grothe E, Moo-Young M and Chisti Y (2008).Fermentation optimization for the production of poly [beta]-hydroxybutyric acid) microbial thermoplastic. Enzyme Microb Tech, 25: 132–141.
13. Gursel I, Yagmurlu F, Korkusuz F and Hasirci V (2002). J. Microencapsulation, 19: 153-164.
14. Gurubasappa. G. Biradar, Shivasharana C. T. and Basappa. B. Kaliwal (2015)
15. Isolation and characterization of polyhydroxybutyrate (PHB) producing Bacillus species from agricultural soil European Journal of Experimental Biology 5(3):58-65
16. Hoagland KD, Rosowski JR, Gretz MR and Roemer SC (1993). Diatom extracellular polymeric substances: Function, fine structure,chemistry, and physiology. J.Phycol, 29: 537-566.
17. Kulpreecha S, Boonruangthavorn A and Meksiriporn B (2009).Inexpensive fedbatch cultivation for high poly (3-hydroxybutyrate) production by a new isolate of Bacillus megaterium. J BiosciBioeng, 107: 240–245,
18. Law J and Slepecky RA (1969). Assay of polyhydroxy butyric acid. In journal of Bacteriology, 82: 52-55.
19. Lee SY and Chang HN (1995).Production of poly (hydroxyalkanoic acid).Adv.Biochem. Engineer. Biotechnol. 52: 27-58.
20. Lin C, Yu RC and Chou C (2004).Susceptibility of Vibrio parahaemolyticus to various environmental stresses after cold shock treatment. Int. J. Food Microbiol.92:201215
21. Looijesteijn PJ, Boels IC, Kleerebezem M and Hugenholtz J (1999).Regulation of exopolysaccharide production by Lactococcuslactissubsp.cemoris by the sugar source.Appl.Environ.Microbiol. 65:5003-5011.
22. López-Cortés and L.A. Lanz(2008).GarcíaMJQ.Microb.Ecol. 56: 112-120

*March-April 2020*
*ISSN: 0193-4120 Page No. 5468 - 5479*

23. Lowry OH, Rosebrough NS, Farrand AL and Randall RJ (1951).Protein measurement with folin phenol reagent, J.Biol.chem. 193: 263-275
24. Misra SK, Valappil S, Roy P and Boccaccini AR(2006). Biomacromolecules,7: 2249-2258
25. Muller HM, Seebach D (1993).Poly (hydroxyalkanotes) A fifth class of physiologicallyimportant biopolymer? Angew. Chem. Int. edit (English), 32:477-502
26. Nallusamy et al.(2013)concluded that, Pseudomonas sp.produced maximum biopolymer among the isolates Panteo agglomerans, Bacillus subtilis, Enterobacter sp.and Pseudomonas sp. which were isolated from some Brassica plants such as cabbage, cauliflower and lettuce.
27. Nicolaus B, Lama L, Esposito E, Manca MC, Importa R, Bellitti MR, Duckworth AW and Grant WD (1999). spp. able to biosynthesize exo- and endopolymers. Journal Industrial Microbiology and Biotechnology,23: 489-496.
28. Oliveira I, Sousa A, Valentão P, Andrade P, Ferreira ICFR, Ferreres F and Bento A, Seabra AR, Estevinho L, Pereira JA (2007). Hazel (Corylusavellana L.) leaves assource of antimicrobial and antioxidative compounds. Food Chem., 105, 1018– 1025
29. Applied and Industrial Microbiology, 8: 578-592.
30. Shankar CVS, Malar A, &Punitha SMJ (2010).Antimicrobial activity of marine bacteria associated with Polychaetes. Bioresearch Bulletin, 1: 24-28.
31. Shivakumar S (2012). Accumulation of poly(3-hydroxybutyrate) by MicrobacteriumbarkeriDSM 20145. Turk J Biol 36: 225–232.
32. Soam A, Singh A.K, Singh R and Shahi S.K (2012). Optimization of culture conditions for bio-polymer producing Bacillus mycoides (WSS2) bacteria from sewage. Current Discovery. 1(1), 27-32.
33. Steinbuchel A (1991).Polyhydroxyalkanoic acids.InBiomaterials.Novel Materials from Biological Sources, pp.123±213.Edited by D. Byrom. Basingstoke: Macmillan
34. Sutherland IW (1983).Microbial exoplysaccharides.Their role in microbial adhesion in aqueous systems.CRC Crit.Rev.Microbiol, 10: 173-201l
35. Sutherland IW (2001).Microbial polysaccharides from gram-negative bacteria.Int Dairy J 11:663-674.
36. , Tanamool V, Imai T, Danvirutai P and Kaewkannetra P (2013). Biosynthesis of polyhydroxyalkanote(PHA) by Hydrogenophaga sp. Isolated from soil environment during batch fermetaion . Journal of Life Science, vol. 5, no. 12. Pp. 1003-1012, 2011
37. Tekin E, Ateş M and Kahraman O (2012). Poly-3-hydroxybutyrate producing extreme halophilicarchaeon: Haloferaxsp. MA10 isolated from CamaltıSaltern, İzmir. Turk J Biol, 36: 303–312.
38. Thombre AG and Cardinal JR (1990). Biopolymers for controlled drug delivery.In: Encyclopedia of pharmaceutical technology. .32: 61-88
39. Uhlinger, D. J. and D. C. White. 1983. Relationship between physiological status and formation of extracellular polysaccharide glycocalyx in Pseudomonas atlantica. Appl. Environ. Microbiol. 45: 64-70.
40. Vandamme E, De Baets S, Steinbuchel A (2004).Polysaccharides I and II.In:Biopolymers.Steinbuchel A, (seies editor). WILEY-VCH; 16: 1694-1700.
41. Volova T (2004).Polyhydroxyalkanoates, Plastic Materials of the 21st Century: Production, Properties and Applications, Nova Science Publishers, New York, NY, USA .22: 412-421.
42. Viju N, Satheesh S and Vincent SGP (2014).Antibiofilm activity of coconut
43. (cocosnuciferalinn) husk fibre extract, Saudi.J.Biol.sci, 20(2013) 85-91
44. Williams AG and Wimpenny JWT (1977).Exopolysaccharide production by Pseudomonas NCIB 11264 grown in batch culture. Journal of General Microbiology,102: 3-21.
45. Yuksekdag ZN, Aslim B, Beyatli Y and Mercan N (2004).Effects of carbon and nitrogen sources and incubation times on poly-beta-hydroxybutrate synthesis by Bacillus subtilis25 and Bacillus megaterium 12, African Journal of Biotechnology,3: 63-66.