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Research Paper

Screening Of Bacteria for Production of Polyhydroxybutyrate (Phb) From Inexpensive Substrate

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ABSTRACT
The purpose of this study was to isolate Polyhydroxybutyrate producing bacteria cultivated from various soil samples, composting, spoiled fruits, curd, dumping site, farm soil were in different areas of Sadra, Gujarat, India. Isolation was done by using nutrient agar medium. 30 different Bacterial Culture isolates were observed by using molasses as a carbon source. Bacterial cultures were perform3d by morphology, microscopy and biochemical IMVIC test, Sudan black b tests. The associated metabolic activity of bacterial culture BRSN$_7$ led to maximum PHB production 0.75g% within 24 hours. These PHB are useful in contrast to synthetic carbon-based polymers. However, the maximum production price limits their commercialization. Focused on optimizating of culture conditions for maximum PHB production in order to reduce the production cost. Culture conditions for these organisms were optimized by changing the parameters, viz., incubation time, pH, carbon source and nitrogen source, inoculums size, temperature. Maximum production of PHB was obtained using 3 % sugar cane molasses as the sole carbon sources resulting in a dry cell mass of 0.85gm % cell dry matter and highest PHB production observed by using Ammonium oxalate as the nitrogen source. The amount resulted in 0.5gm % and 16% inoculum size. The highest PHB production as 0.7gm % and The Results obtained with 0.78gm% maximum PHB production at 7 Ph show show 0.825 g% maximum PHB production at a temperature of at 30^{0}C temperature. Therefore, we used optimized culture conditions were used to culture the isolates for extraction of PHB and its analysis. The compounds extracted by FTIR-analysis show characteristic peaks of hydroxyl and carbonyl groups, and are present with CH$_3$ bends.

Keywords - *molasses, culture, optimization, Polyhydroxybutyrate (PHB)*

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I. INTRODUCTION

Plastic is a synthetic polymer with versatile strength, durability and resistance to deterioration. They are an important part of almost every industry that has replaced the glass and paper in packaging but now they have these very beneficial properties. Have now several drawbacks like world's biggest environmental problem. The Accumulation of unwieldy plastics in the environment has become a global problem. Microorganisms in the environment are not able to break down plastics due to their chemical complexity. Incineration of plastics is potentially dangerous and generates a surplus amount of CO_2, along with some highly toxic gases that are potentially dangerous and cause global warming and air pollution and health hazards. During the combustion of plastic waste, hydrogen cyanide can be formed from acrylonitrile - based plastics and may cause potential health hazards. It can be recycled but it is very expensive and has high energy value. The sorting of the wide variety of discarded plastic waste is also a very time-consuming process (Khanna and Srivastava, 2005). All of these activities lead to imbalance between our ecosystem and biological system. Proper disposal of synthetic plastics is a major issue, as these are xenobiotic in nature and hence resistant to microbial degradation processes. Synthetic plastics persist in our ecosystem for several years in our ecosystem. This is main reason for the resistance to microbial degradation (Rao *et al.*,2019).

Polyhydroxyalkanoates re 100 % biodegradable polymers only and are accumulated intracellular in the form of granules by many micro-organisms usually during the stationary phase in the presence of essential nutrients and in the presence of excess carbon sources. Increase in addition to biodegradability and biocompatibility, These microbial polyesters exhibits low crystalline, increased impact strength and flexibility in addition to its biodegradability and biocompatibility nature (Das *et al.*,2019). Bioplastic are gaining more and

more attention these Days due to their diverse application and rapid biodegradation. The renewable carbon sources are used in the PHAs manufacturing process, this is safer and reduces the spread of pollutants. Degradations of Polyhydroxyalkanoates degradation needs approximately 5 to 6 week time period in a microbial media to completely convert into water and carbon dioxide which provide the benefit to the environment. This is because PHA does not allow air and water to pass through. thus they are appropriate for films and coatings production industries (Amini *et al.*, 2020)

Polyhydroxybutyrate belongs to the family of biodegradable polymers family of PHA. It has mechanical properties very similar to petroleum based plastic (Li *et al.*, 2020). Polyhydroxybutyrate is a short-chain biopolymer multiple characteristics and can be easily degraded aerobically and an aerobically. Due to its biodegradability and other excellent properties, PHB can be used in medicine and tissue engineering (hard and soft tissue implants), intelligent drug delivery systems, food packaging and agriculture due to its biodegradability and other remarkable properties. In addition to its biodegradability, PHB is biocompatible as well. However, the high manufacturing costs of PHB severely limits the biomedical and environmental uses of this chemical (Ghoddosi *et al.*, 2019).

PHB is synthesized from acetyl-CoA produce by the bacteria in sequential action of three enzymes (1) 3 – ketothiolase (phbA gene) (2) acetoacetyl – CoA reductase (phbA gane) (3) PHB Synthase (phbC gene).PHB production is mainly based on renewable resources (Shah *et al*., 2008).

PHB has also been reported to increase production by increasing the ratio will increase its production. The initial sugar concentration affects the results of high CDW and PHB content. Approximately 45% of total (PHB) production expense is the cost of carbon sources like glucose or sucrose. Some research has attempted to attenuate production costs, as an example by the use of different carbon sources for the growth of low-cost bacteria. Efforts are made to scale back costs by using renewable and renewable, low-cost carbon sources. PHB commercial production was used has been using relatively cheap substrates such as methanol, beet molasses, ethanol, starch and whey, cane molasses as a sole carbon source, wheat hydrolysate and fungal extract or soy cake (Chaijamrus and Udpuay, 2008).

Molasses is a nutritional ingredient rich in trace elements such as sugars, proteins, amino acids, Mg, P and K, and has proven to be an excellent alternative sugar production process at low cost. Vinasse, another residue from the ethanol distillation process, is rich in salt. Therefore, it can be used as an aid to the refinement of the medium for the fermentation process**.**

II. Materials And Methods

2.1 Collection of samples

Soil sample were collected aseptically from dumping site, Root nodules of leguminous plants, garden soil and water from various sites like paper pulp industrial waste, house hold water, sewage water, salt water and contaminated fruits and curds.15 -20 g of soil samples were collected by scrapping to depth of 5-8 cm with a sterile spatula. The samples were placed in sterile plastic bags stored at 4^0 C and transported to the laboratory.

Fig: 1 and 2 Soil and Molasses

2.2 Isolation of PHB producing organisms from soil Sample.

One gram of soil sample was suspended in 99 mL sterile distilled water and shaken vigorously for 2 min. Then diluted soil sample was serially diluted and plated on nutrient agar medium. The plate was incubated at 37 ^{0} C for 24-48 hrs**.** Kesaulya et al., (2021)

2.2.1 Characterization of isolates

Morphological characterization: The identification of isolates consisted firstly in macroscopic (colony, size, margin, elevation, surface, consistency, opacity, pigmentation) and microscopic. Selected isolates were investigated according to Bergey's manual of Determinative Bacteriology by growing them on nutrient agar medium.

2.2.2 Microscopic observation of PHB granules by Sudan Black-B dye

The PHB producing bacteria was further Sudan Black B staining method; Sudan black B stain was prepared as 0.3% solution (w/v) in 60% ethanol. The smear of culture was made on glass slide and heat fixed. They were

stained for 10 min with Sudan Black-B solution, rinsed with water and counter stained with 0.5% safranin for 5 min and observed under light microscope at 100x magnification.

2.2.3 Biochemical characterization: The identification of the bacterial isolates on the basis of biochemical test, different test were performed in selected bacteria catalase test, indole production test, citrate utilization test, urease, starch hydrolysis test, methyl red test, etc. (Shrivastav *et al*., 2010)

2.3 Profile of molasses

Molasses was collected from the sugar factory and store at the room temperature 37 ^{0} C. Molasses sample was analyzed for physicochemical parameter such as pH, potential (mV) was measured by using digital pH meter (Systronics MK VI), Total solids (TS), Total volatile solids (TVS), Chemical Oxygen Demand (COD), Sulphate, Phosphate, anthrone, DNSA (APHA 2017).

2.4 Inoculum development

PHB producing organism was grown on nutrient agar slant at 37 ^{0} C for 24h. The full grown slant were stored at 4 ^{0} C and were subculturing in every two weeks, a volume of 100mL of BHM broth taken in 250 mL Erlenmyer flask was inoculated a loop full of cell from old culture and kept at 37 ^{0} C on a incubator, after 24h of incubation,1 O.D. at 600 nm (approximately 1* 10^7 cell/mL), BHM broth was used as inoculums (Prajapati *et al*., 2014).

2.5 Media composition for PHB synthesis

A liquid culture medium containing 5% molasses (v/v) as carbon source $MgSO_4$ 0.2; $CaCl_2$ 0.02; KH_2PO_4 1.00; K_2HPO_4 1.00; NH_4NO_3 1.00; $FeCl_3$ 0.05; (pH 7.0 ± 0.2)] was sterilized, after add the bacterial culture. The flasks were kept at 30ºC with 120 rpm in shaker incubator for a week, (Ganapathy *et al*., 2018).

2.6 PHB extraction from cellular biomass

Cell-biomass was harvested from the production broth by using centrifugation at 10,000 rpm for 15 min at room temperature. The pellet was washed with acetone and distilled water followed it, PHBs extraction was carried out using chloroform at 37 ºC overnight at 200 rpm on rotary shaker. The cellular debris was removed by filtration through Whatman no. 1 filter paper. The extract was concentrated by evaporating chloroform in rotary evaporator and precipitated by drop wise addition of double volume of chilled methanol. Precipitation step was performed twice to obtain purified PHBs. PHB extraction method was adopted using well established methods. Some step of this method modified by me and my guide. (Purama *et al*., 2018, Ray *et al*., 2016). Each isolated bacterial strain was checked for PHB production and the isolate showing maximum PHB production was identified and selected for further production optimization.

2.7 Optimization study for maximum PHB synthesis

Among the bacterial isolates, optimization of PHB production as it gave a higher PHB production in a preliminary experiments, for the fermentation process optimization, all experiments were carried out in200 ml in 500 mL Erlenmeyer flasks and different response like cell dry weight (g/L) and PHB dry weight (g/L) were recorded (Liu *et al*., 2011).

2.7.1 Effect of inexpensive carbon source (molasses) on PHB

Effect of different concentration sugarcane molasses 1 to 5% were added in a production medium(MSM), then 10% culture was added in medium and incubated at 37 ^{0} C on shaker with170 rpm for 48 h, based on maximum PHB yield the best concentration as carbon source.

2.7.2 Effect of different nitrogen source on PHB production

The PHB positive isolate was inoculated in broth containing the best concentration of carbon source and different nitrogen source peptone, ammonium sulfate, ammonium oxalate, ammonium molybdate, yeast extract, ammonium chloride separately, PHB yield was determined after 48 hrs incubation at 37 ^{0} C in shaking condition.

2.7.3 Effect of inoculums size on PHB production

Different inoculum size were prepared to study of the maximum PHB production so 8, 10, 12, 14, 16, 18(%) inoculums size were prepared, thereafter separately inoculated in MSM medium for 37^0 C for 48h under shaking condition at 170 rpm, to attain maximum biomass for PHB. After incubation period, culture was centrifuged and estimate to PHB.

2.7.4 Effect of pH on PHB production

To standardize the optimum pH for the production of PHB, the bacterial cultures were inoculated in MSM medium at different pH (6.5, 7,7.5,8and 8.5) the media pH was adjusted with hydrochloric acid and sodium hydroxide and incubated at constant temperature 37^0 C for the 48 h. The optimum pH was determined based on the maximum amount of PHB produced.

2.7.5 Effect of temperature on PHB production

The standardize the optimum temperature for PHB production, bacterial isolated in broth at temperature for 48 h. PHB yield was quantified based on that optimum temperature for maximum amount of PHB produced.

2.7.6 Effect of incubation time on PHB production

PHB producing isolated was incubated for a different time period of 96h, at each point of time (24, 48, 72 and 96 h) culture was tested for bacterial growth and PHB production by dry cell weigh measurement.

2.8 Analytical technique

2.8.1 FTIR analysis

The PHB extracted from the most potent PHB producing isolated was analyzed by FTIR spectroscopy using as spectrum with scanning as a spectrogram between 4000 and 400 cm^{-1}. Purified PHB sample (2 mg) was mixed with 20 mg of Potassium Bromide. FTIR spectrum was recorded and confirmed the functional group of the extracted polymer (Ganapathy *et al*., 2018).

III. RESULTS AND DISCUSSION

3.1 Isolation of PHB producing bacterial strain and its characterization

total 30 different strains were isolated from varies site biogas slurry, oil contaminate soil, curd, contaminated fruits, desert soil, were applied to find out the PHB producing bacteria by enrichment culture technique employing nutrient agar medium. Amongst which 4 strains found to effectively accumulate. Based on the dry weight of the extracted PHB and the bacterial isolated $BRSN_7$ was selected as the maximum PHB producer and subjected to further optimization studies.

Table: 1 Highest PHB producing organisms

$BRSN_1$	0.60 gm %
$BRSN_6$	0.55 gm %
$BRSN_7$	0.75 gm %
BRSN9	0.49 gm %

Growth of selected strain $BRSN_7$ after 24h on nutrient agar and in submerged media containing BHM after 24h were subjected to gram's staining and Sudan black B staining respectively. Confirm the present of PHB granules in $BRSN_7$. Staining technique is generally preferred in screening of PHB producer have demonstrated use of Sudan black b fluorescence staining method for the screening of PHB producing bacterial isolates from soil. The colony characteristics of $BRSN_7$ stain are tabulated (Table: 2).

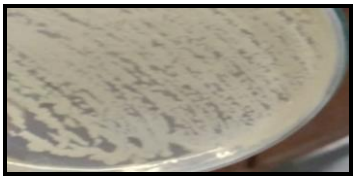


Fig: 3 Colony observation of $BRSN_7$

Table: 2 Morphological characterization

Colony characteristics	**Observation**
size	medium
shape	round
elevation	convex
surface	smooth
Glisteing edge	entire
opacity	translucent
colour	white
consistency	moist
Gram staining	positive

3.2 Characterization of molasses

Physicochemical characterization of molasses analyzed by individual parameters were pH, gravimetric analysis, chemical oxygen demanded, biological oxygen demanded, total Kjeldahl nitrogen, reducing sugar, total sugar, sodium and potassium detected using various methods as shown in Table.

Table: 3 Biochemical characterization

Biochemical test	**$BRSN_7$**
M-R tst	-
V-P test	+
Indole test	-
Urea hydrolysis	_
Oxidase test	+
H_2s production test	-
HCN test	-
Catalase test	+
Citrate utilization	+
TSI	+

Table: 4 Characterizations of Molasses

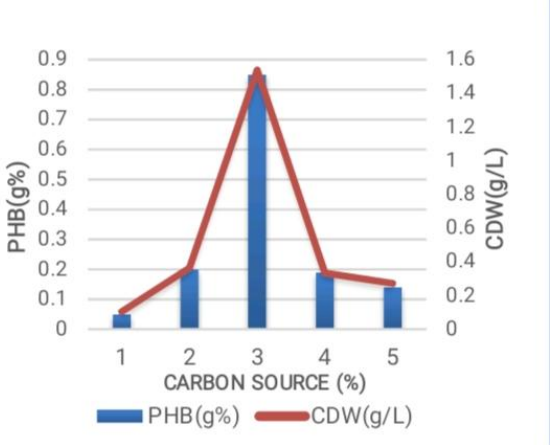
Parameters (units)	**Techniques**	**Molasses**
Ph	-	4.74
mv	-	1.41
Reducing sugar (mg/l)	Dinitrosalicylic acid	12.5
Total sugar (mg/l)	Anthrone	33.31
COD	Close efflux method	6.133×10^4
TS (mg/l)	Gravimetric method	978
TDS (mg/l)	Gravimetric method	177
Sulphate (mg/l)	Turbidometric method	-
Phosphate (mg/l)	Colorimetric method	-

3.3 Optimization of environmental and nutritional parameters for PHB

Optimization of process parameters, namely incubation time, incubation temperature and initial pH of the medium was carried out. Different inexpensive carbon source were checked for the PHB production. All the experiments were carried out in triplicate with 200 mL of production media in 500 mL flask and different responses were recorded like cell dry weight (g/L) and PHB (g/L).

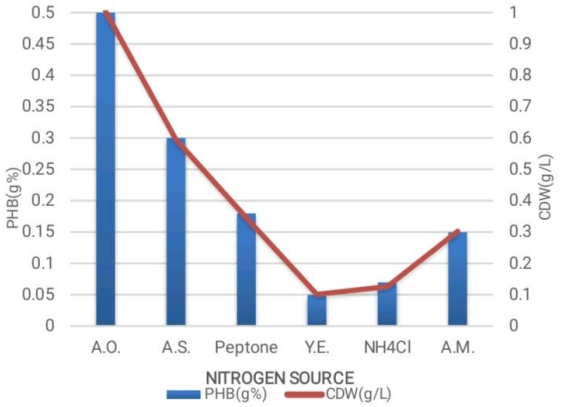
3.3.1 Effect of carbon source on PHB production

In order to find the effect of carbon source for PHB productions, to optimized the effect of carbon source in different concentration 1 to 5% of molasses. our obtain result indicate in 3% maximum PHB production(0.85 gm%) was observed by strain BRN_7. It was noted that produced dry biomass (1.53 g/L) and maximum productivity 24 hours.



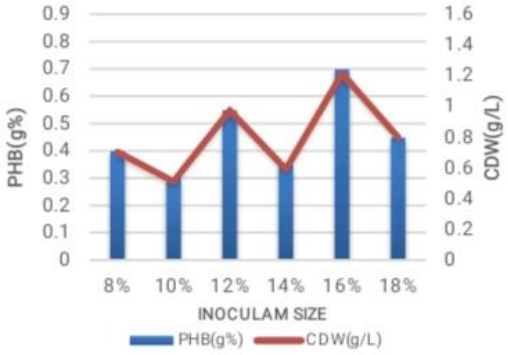
3.3.2 Effect of nitrogen source on PHB production

In order to investigate the effect of nitrogen source for PHB production, the PHB production is greatly influenced by the availability of nitrogen source as it is the ultimate source of fermentation. To optimize the effect of nitrogen source in raw material as use peptone, yeast extract, NH_4Cl, $(NH_4)_2SO_4$, Na_2HPO_4, $NaNO_3$, Ammonium oxalate as a various source of nitrogen. Our obtained results indicated that Ammonium oxalate was found to support the maximum production of PHB (0.5gm%) by strainBRSN7, it was noted that produced dry biomass (1.21 g/L) and maximum productivity of PHB within 24 hrs. It was studied with nitrogen source supplement and enhanced production of PHB.



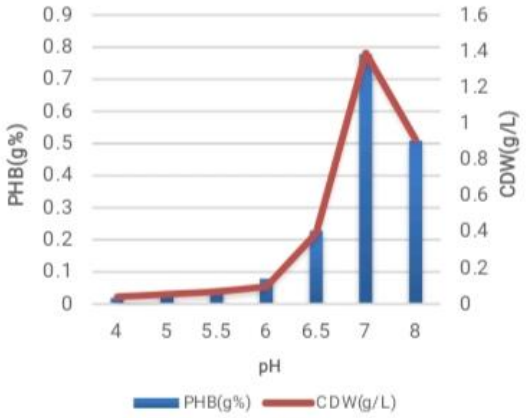
3.3.3 Effect of inoculums size on PHB production

Inoculum size is one of the important factor and the production activities of microorganisms are affected by change in inoculums size, plays an important role because of sufficient cell mass required to successfully convert substrates to product and also increase PHB productivity. Optimization study was carried out with the, BRSN7. The effect of inoculums size on PHB production was evaluated with the addition of different inoculums ranged from 8 to 18.0 % (v/v). Obtained results showed maximum PHB production (0.7 gm %) with BRSN7 produced dry biomass (1.21 g/L) of PHB within 24 hrs at 16% inoculum size within 24hrs. A further increase or decrease in inoculums concentration showed gradually decline in PHB production



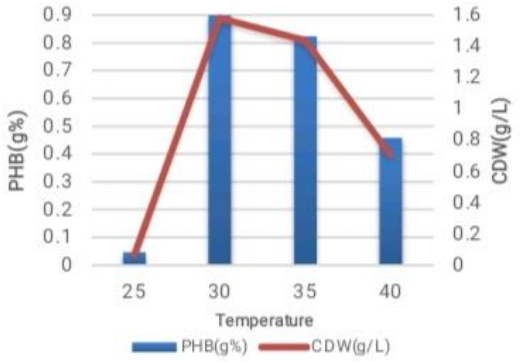
3.3.4 Effect of pH on PHB production

Initial pH of culture medium is one of the key factors affecting PHB productions in fermentation because it provides the acidic condition in acidogenesis phase for high PHB production. pH of the fermentation is greatly affect to production of PHB there for we consider pH as an important parameter during fermentation. 4 to 8 different pH were carried out for PHB production. The obtain results showed maximum PHB production (0.78 gm%) with BRSN7 produced dry biomass (1.38 g/L) of PHB within 24 hrs at 7 ph within 24hrs.



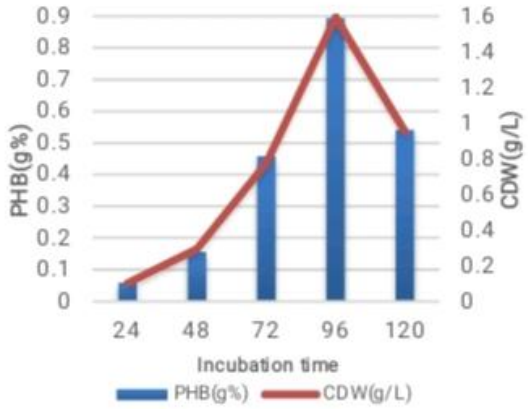
3.3.5 Effect of temperature on PHB production

The influence of temperature for PHB productivity is related to the growth of the organisms. The metabolic productivity is very sensitive to the incubation temperature. In present study, effect of temperature on PHB in fermentation was conducted in arranged at 25to 40 ºC. The obtain results showed maximum PHB production (0.825 gm %) with BRSN7 produced dry biomass (1.43 g/L) of PHB within 24 hrs at 30 ºC Temperature within 24hrs.



3.3.6 Effect of incubation time on PHB production

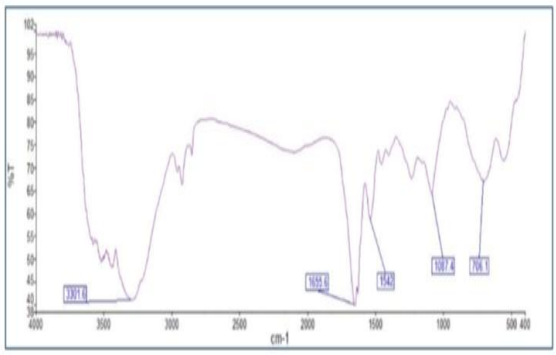
To optimize the end product a classical optimization approach was adopted to study the effect of incubation period on the PHB production. To elucidate the effect of different time period on the production of PHB form BRN_7 on the physicochemical analyze under fermentation, experiment were conduct different periods from 24 to 120 hrs. The obtain results showed maximum PHB production (0.896 gm %) with $BRSN_7$ produced dry biomass (1.59 g/L) of PHB within 96 hrs at 30 ºC.



3.4 Characterization of PHB produced by $BRSN_7$

Characterization of polymer may relevant the chemical and thermal properties of PHB produced by BRN_9 using molasses. For the characterization of PHB produced by, the polymer was subjected to Fourier transformed infrared spectrometry (FTIR) analysis.

3.4.1 FTIR analysis



The obtain result performing FTIR it used for the characterization of polymer by means of infrared light for the detection of chemical groups. PHB produced by $BRSN_7$ was carried out from 400 to 4000 cm^{-1} using the perkin-elmer spectrum Gx. FTIR spectrum of PHB obtain from $BRSN_7$ show the presence of following group.

Table: 5 FTIR spectrum of extracted PHB

cm^{-1}	Represent chemical bond
400-4000	-
706.1	Skeletal (amorphousvibration)
1087.6	-C-O- Stretch
1542	$-CH_3$- bending
1655	Carbonyl group
3301.6	-OH hydroxyl stretch

IV. CONCLUSTION

The aim of the research was PHB production by bacteria isolated from various soil samples. For the fulfillment of the research objective we started our study for isolation of bacteria from collected various samples like compost pit, composting, spoiled fruits, curd, dumping site, farm soil. Sample was first cultivated in nutrient agar medium and screening procedure. The well grown bacterial cultures were studied for PHB production under mineral salt medium. 30 different bacterial Culture were obtained from the various collected samples. Preliminary identification of Potent bacterial culture was done by morphologically and microscopic observation. The screening of bacterial culture done by PHB experiments tested 30 bacterial cultures $BRSN_7$selected for highest PHB production. Amongst all, BRN_7 was chosen for highest PHB production 0.75 g%.

Molasses characterization in term of pH, COD, BOD, TS, phosphate, sulphate, TVS was studied. In our study we have followed airlift fermentation method to justify our goal. Since the production of PHB was in low concentration, attempts were made to increase the production of PHB by optimizing the growth condition in mineral salt medium.

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