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Media optimization for Phb production and its application as precursor for bioplastics

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Abstract
In the present study, PHB, which is used as precursor in production of biopolymer, has been fermentatively produced. The microbial source used is *Alcaligenes spp* NCIM 5085. The media optimization was performed. Few media parameters such as carbon source, nitrogen source, citric acid concentration have been optimized for cheap and higher production of PHB. The PHB produced has been isolated, purified and detected by TLC and FT-IR. The present study shows that sucrose is best carbon source for the production of PHB. Alternatively Cane molasses can be used as a raw material for reducing the cost of production which gives PHB of concentration comparable to sucrose.

Keywords: PHB, TLC, FT-IR, Media optimization.

1. Introduction

Poly 3-R-hydroxybutyrate (PHB) is a bacterial intracellular polyester compound of 3-hydroxybutyric acid [1]. It is alternative source for petroleum derived polymers used for making plastic since PHB can undergo biodegradation at various environmental conditions i.e., aerobic, anaerobic [2], thermophilic [3]. PHB is produced by variety of microorganisms, including *Alcaligenes eutrophus,* [4] *Bacillus megaterium* [5], etc. which is accumulated as discrete granules upto levels as high as 90% of cell mass [6]. Generally, production of PHB is enhanced by nutrient limitation i.e. nitrogen, oxygen, phosphorus [7]. *Alcaligenes latus* has been known to produce PHB in growth associated manner even under nutrient sufficient conditions [8]. PHB has many applications in medicine, veterinary practice, tissue engineering materials, food packaging, and agriculture [9]. The only barrier for the commercialization of PHB to be used in its wide applications is their high production cost [10]. Hence, to reduce its cost of production, it is important to produce it with high productivity. Various cultivation strategies involving inexpensive renewable carbon substrates are being explored for making the cost of production lower and get high productivity [11]. Hence, the main aim of our study is optimization of fermentation process by different media parameters for PHB production so as to reduce its cost and get maximum production at industrial level.

2. Materials and Methods

Organism used: *Alcaligenes spp* NCIM 5085 was used for production of PHB. The cultures were revived on Nutrient Agar Medium and it was maintained and sub cultured every 30 days on the fresh Nutrient Agar Medium.

Fermentation process: PHB fermentation was carried out in 2 stages. In the first step culture was enriched on Growth Medium. Beef Extract Medium was used as Growth Medium containing (g/l) Beef Extract-10gm, Peptone-10gm, NaCl-5gm, and pH-7.2. [12]. The culture was inoculated in medium and incubated for 24 hrs at Room temperature (RT) for 160-180 rpm on Rotary Shaker. The inoculum was transferred to the Production Medium containing (g/l); KH_2PO_4 - 1.5g, $Na_2HPO_4.12H_20$ - 9g, $MgSO_4.7\ H_2O$ -0.2g, $CaCl_2.2\ H_2O$-0.01g, Citric acid-2g,(Trace element solution -1ml ($FeSO_4.7\ H_2O$, H_3BO_3, $ZnSO_4.7\ H_2O$, $MnCl_2.4\ H_2O$, $(NH_4)_6Mo_7O_{24}.4\ H_2O$, $NiSO_4.7\ H_2O$, $CuSO_4.5\ H_2O$) Carbon source (Sucrose)-20gm, Nitrogen source $(NH_4)_2SO_4$ -3gm. [13]. The above media was incubated for 4-5 hrs at RT for 160-180 rpm on Rotary Shaker. For media optimization, production was carried out by changing the carbon source for maximum turbidity. Carbon source used were Sucrose, Glucose and Fructose and its concentration were changed accordingly. The production media was again optimised changing the Nitrogen source (Ammonium Sulphate) concentration and Citric acid concentration.

Extraction of PHB: Further recovery process was carried by taking the fermented media and centrifugation was done at 10,000rpm for 10mins. Then the supernatant was discarded and the cell pellet obtained was suspended into Chloroform and cold shock treatment was given for 1 hr at -20 ^{0}C. The treated pellet was suspended into Chloroform: Methanol: Water (65: 25: 10) solvent mixture in a separating funnel and vigorous shaking was done for 10 minutes. Then allow to stand till two separate layers are seen and collect the lower layer in a pre weighed Petri-dish and allow the Chloroform to evaporate. A thin transparent film is observed.

Analysis of PHB

The PHB production was analysed by using TLC for lipid analysis [13] and the PHB granules formed by solvent extraction were scraped to get dry powder which was used to prepare KBr discs and subjected to FT-IR [14].

3. Result and Discussion

Detection by TLC: The Chromatography Chamber was saturated with solvent system. The sample granules were dissolved in Chloroform and spotted on TLC paper by capillary.

The TLC paper is placed in saturated chromatography jar and allowed to run. When the mobile phase reached 3/4th length of paper, the paper was taken out and air- dried. TLC paper was exposed to iodine vapours. The brown spot observed indicated the presence of lipids [13]. PHAs are primarily linear, head-to-tail polyesters composed of 3-hydroxy fatty acid monomers [16]. Hence, from TLC, presence of lipids indicated the granules of PHB.

Ftir analysis: Spectra for sample granules were recorded between 40-4000 (cm^{-1}) using Spectrum65 FTIR spectrometer (PerkinElmer). A broad peak at 3424.09 (cm^{-1}) indicates the presence of –OH group at 2925.97 (cm^{-1}) indicates methylene, at 2854.49 (cm^{-1}) indicates methine and at 1734.42 (cm^{-1}) indicate ester linkage i.e. C=O bond whereas methyl group is confirmed by a small peak at 1261.64 (cm^{-1}). Also, the 1651(cm^{-1}) band is characteristic to conjugated C-C bond [14, 15] the same peaks were observed in our samples which confirm the PHB granules (Fig.1).

Effect of Carbon Source: The carbon substrates were varied to check the maximum utilization by the bacteria. Sucrose, Glucose and Fructose were used. The effect was seen in terms of turbidity observed at the end of incubation period. The effects are shown in Table No.1.

Table 1: Effect of carbon source

Carbon Source	Turbidity
Sucrose	++
Glucose	–
Fructose	+

Where, – No turbidity, + less turbidity, ++ maximum turbidity.
The turbidity indicates the cell mass and thus indicates the PHB contents indirectly.

Effect of concentration of Carbon Source: To determine the optimum amount of the substrate, Sucrose was used as carbon source. The PHB obtained per 100ml were checked at 2%, 3%, and 4%. The amount of PHB was calculated by dry weight determination as shown in Table No. 2.

Table 2: Effect of Sucrose concentration

Sucrose concentration (in gm %)	Dry Weight (mg)
2	27
3	35
4	20

The maximum amount of PHB was produced at 3% sucrose concentration.

Effect of concentration of Nitrogen source:
The nitrogen source used in medium was Ammonium sulphate. The different concentration of Ammonium sulphate was used in medium as described in Table no. 3.

Table 3: Effect of Ammonium sulphate concentration

Ammonium sulphate (in gm %)	Dry Weight (mg)
0.1	00
0.3	07
0.5	11

There was no PHB produced in medium containing 0.1% ammonium sulphate whereas maximum PHB production was observed at 0.5% ammonium sulphate in medium.

Effect of citric acid concentration
The citric acid concentration used in medium was 0.1%, 0.2% and 0.3% for media optimization shows no significant change in PHB granule production.

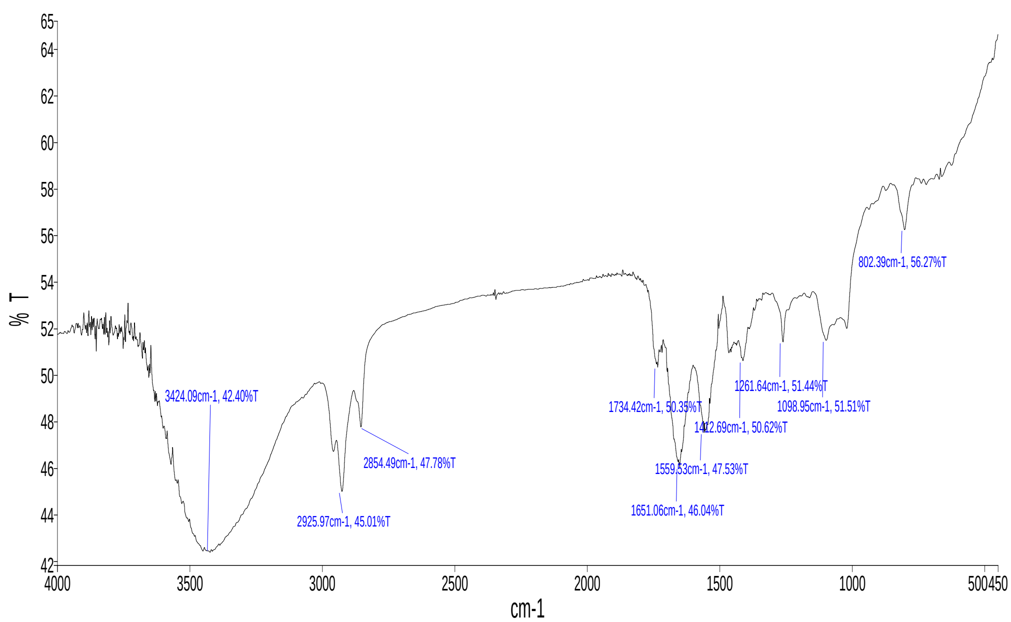

Fig 1: FT-IR Results of PHB

4. Conclusion
From present investigation it was concluded that sucrose is the best carbon source for production of PHB. Medium as described above for production containing 3% sucrose 0.5% Ammonium sulphate can be used for maximum PHB production. Hence, similar conditions can be used at large scale production of PHB and its utilization as precursor for bio-plastic can be made more common so as to overcome the problems associated with use of petroleum- derived plastic. The PHB production can be maximized in future aspects by strain

improvement and further media optimization by using different parameters also.

5. References

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