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Original Research Article

Optimization of cultural conditions for fermentative production of Polyhydroxybutyrate from Marine bacteria

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Abstract

Polyhydroxybutyrate (PHB) have been drawing much attention as biodegradable substitutes for conventional non-biodegradable plastics and recommended for application in various industries. The aim of the present work was to study the production of Poly-3-hydroxybutyrate (PHB) under nitrogen limited conditions by marine Bacteria isolated from the coastal area of Kodur near Nellore district. An attempt has also been done to study the effect of various process parameters on PHB production such as best carbon, nitrogen sources and their concentrations, NaCl tolerance, temperature and p^H of the medium. Results from the study showed that Glucose and yeast extract in the ratio of 2.5:0.2 is more suitable for PHB production. The PHB yield was more optimum at 30^0C temperature, neutral pH and 2% NaCl concentration favoured the fermentative production of PHB. Microbiological examination has revealed that the marine isolate was identified as *Bacillus amyloliquifaciens* by 16s rRNA sequencing.

Key words: Marine bacteria, Optimization of cultural conditions-Polyhydroxybutyrate production

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1.0 Introduction

Bioplastic is defined as a form of plastic synthesized from renewable resources such as plant starch and microbial species. To combat challenges due to environmental pollution caused by synthetic polymers, many attempts are being made to discover bioplastics which are environmental friendly. Among various bioplastics which are being exploited, Polyhydroxybutyrate (PHB) is gaining popularity [1]. It is an intracellular lipid reserve material accumulated by many bacteria under the conditions of nutrient stress. It is primarily a product of carbon assimilation and is employed by microorganisms as a form of energy storage material [2]. The optimal conditions for PHB production by microorganisms usually include an excess of carbon source and exhaustion of single nutrient such as Nitrogen, Phosphorous, Sulphur or Oxygen.

PHB has a promising market potential for due to their water resistance, good tensile strength and oxygen permeability properties. PHB has good resistance to ultra-violet radiation but poor resistance to acids and bases [3]. PHB is water insoluble and relatively resistant to hydrolytic degradation which differentiates PHB from most other currently available biodegradable plastics like polyhydroxyvalerate and polyhydroxylactate, which are water soluble and moisture sensitive. PHB has been found as eco friendly substitute because it can easily biodegrade aerobically and anaerobically by variety of bacteria and can also be produced from renewable resources [4]. PHB is being touted as an alternative source of plastics due to its similar physical properties like Polypropylene and useful properties [5]. Inspite of many useful properties, it has not been commercialized for large scale application due to it's high cost of production and less efficient recovery methods. Marine organisms have strategies to produce novel compounds like PHB due to the unique environment they survive. Hence, the present research work has been aimed to use marine microorganisms as biological tools for production of PHB.

A generic process for PHB production by bacterial fermentation consists of three basic steps: fermentation, isolation and purification. The factors affecting the PHB production in the fermentation medium include best carbon and nitrogen sources and their concentrations, NaCl conccentration, pH and temperature [6]. Hence, an attempt is also being made in the present work to optimize the fermentative conditions which favour the production of PHB.

2.0 Materials and methods

2.1 Collection of marine samples

Marine samples are collected from coastal area of Kodur near Nellore district, Andhra Pradesh under aseptic conditions and brought to the lab for isolation of marine bacteria.

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Figure-1: Bluish black PHB granules

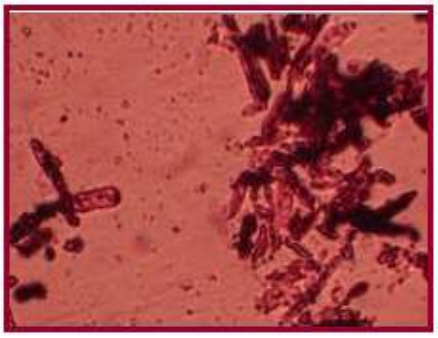

Figure-2: PHB granules in Phase-contrast microscope

2.2 Isolation of marine bacteria

Marine samples were subjected to 10 fold serial dilution ranging from 10^{-1} to 10^{-8} and plated on marine agar medium and nutrient glucose agar medium. After seven days of incubation, microbial isolates obtained were purified, sub-cultured and maintained on marine agar and nutrient glucose agar slants at 4^0C.

2.3 Screening of marine isolates for PHB production

The isolates were further screened for PHB production by Sudan Black B staining method [7]. In Sudan black B method, microbial isolates were stained with Sudan black B (0.3%, w/v) for 5 to 15 minutes and then immersed in xylene. Finally counter stained with aqueous safranin (0.5%, w/v)

for 30 seconds. The PHB granules in microbial isolates appeared as blue black droplets and cytoplasmic part of micro organisms appeared as pink under oil immersion objective lens.

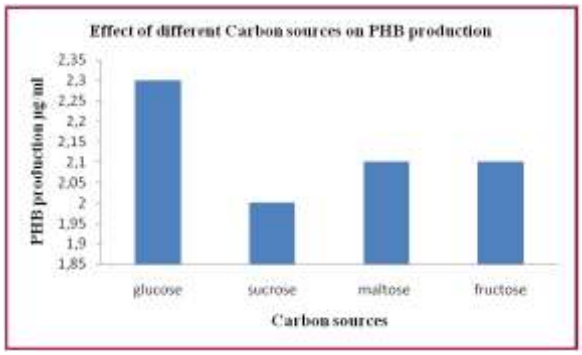

Figure- 3: Effect of different Carbon sources on PHB production

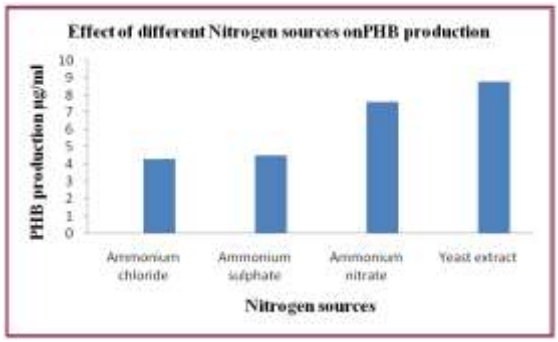

Figure-4: Effect of different Nitrogen sources on PHB production

2.4 Characterization of potent marine isolates

Selected PHB producing bacteria was stained by standard gram staining procedures and observed under microscope for their morphological characterization. Biochemical tests namely Indole production, Methyl Red test, Voges Proskauer, Citrate utilization test, Urease test, Oxidase test, Catalase test, Carbohydrate fermentation and Nitrate Reduction tests were performed to know their biochemical properties.

2.5 Sequencing

The positive marine isolate subjected to molecular characterisation [8] by 16srRNA sequencing and submitted to Genbank.

2.6 Fermentative production of PHB

The PHB producing strains, selected by primary screening were subjected to PHB production in Nitrogen limited medium by testing the effect of various media ingredients like carbon and nitrogen sources on PHB production [6]. The best carbon and nitrogen sources for PHB production was determined simply by replacing one carbon source with other cabon sources (glucose, sucrose, fructose and maltose at 1% level) and nitrogen source with other nitrogen sources (ammonium chloride, ammonium sulphate, ammonium nitrate and yeast extract at 1% level respectively).

Effect of different concentrations of best carbon source (1.0, 1.5, 2.0, 2.5g/100ml) nitrogen source (0.2, 0.4, 0.6, 0.8, 1g/100ml), C:N ratio (1:0.2, 1.5:0.2, 2:0.2 and 2.5:0.2) and NaCl concentration(0.5, 1.0, 1.5, 2.0, 2.5 g/100ml) were determined similarly.

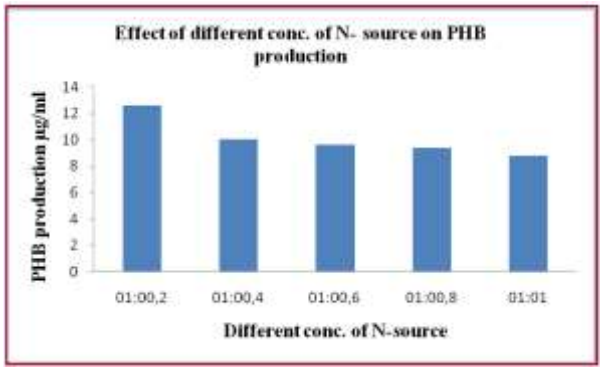

Figure-5: Effect of different concentration of Nitrogen source on PHB production

After optimising the carbon and nitrogen sources, different pH of the media were maintained (5.0, 6.0, 7.0, 8.0) and different temperatures (20^0 C, 30^0 C, 40^0 C and 50^0 C) are tested to know the optimal conditions for PHB production. After 48 hours of incubation, PHB yields were quantified spectrophotometrically by Sodium Hypochlorite assay method.

2.7 Estimation of PHB from fermentation samples by Sodium Hypochlorite assy

10 ml of suspension of culture was centrifuged at 6000 rpm for 15 min.Then the pellets were suspended in 5 ml of sterile water and dried for 24 hrs at 100^{0}C.To cell suspension, 5 ml of Sodium Hypochlorite solution was added and incubated at 60^{0}C for 1 hour [9]. Centrifuge the suspension at 6000 rpm for 15 min and the supernatant was separated. To extract cell lipids and other molecules (except PHB) from supernatant, 5 ml of 96% (1:1 v/v) ethanol and acetone were added. Now 10 ml of chloroform was added to the tube by placing it in hot water bath (60^{o}C). Chloroform was evaporated to obtain PHB crystals.10 ml of 98% H_2SO_4 was added at 60^{0}C and kept for 1hr to convert PHB crystals into crotonoic acid. After cooling to 25^{0}C, the amount of

PHB was determined spectrophotometrically at 235nm against H_2SO_4 as blank [10].

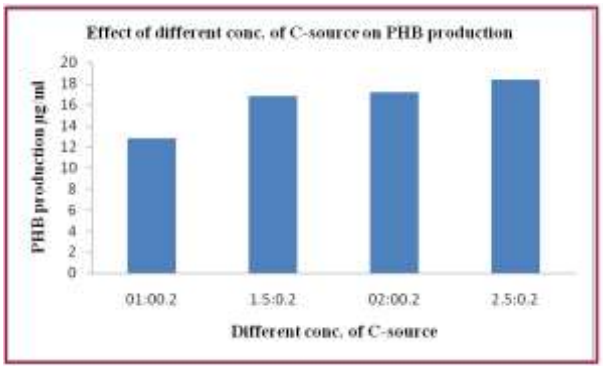

Figure-6: Figure-5: Effect of different concentration of Carbon source on PHB production

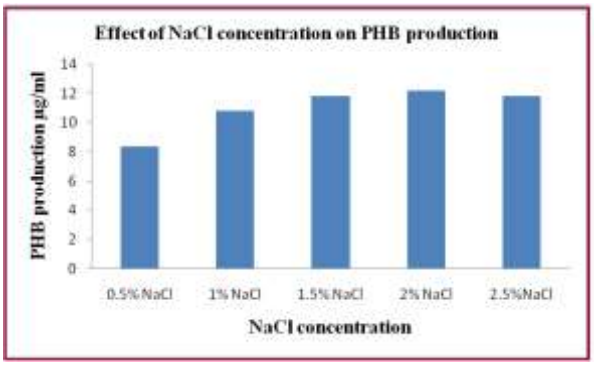

Figure-7: Effect of different concentration of Sodium Chloride on PHB production

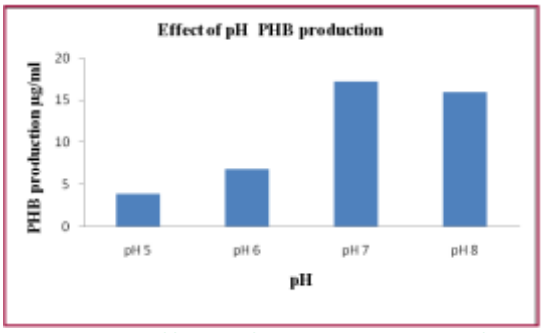

Figure-7: Effect of pH on PHB production

3.0 Results and discussion

The harmful effects of synthetic plastics in the environment have been increasing for the last several years. This ecological awareness impelled development of new, eco friendly materials, especially for single use plastic items. Polyhydroxybutyrate [9] is an alternative to synthetic plastics because it is easily biodegradable. The use of biodegradable plastic [4] appears to be a promising solution to the problems due to the use of synthetic plastics.

Polyhydroxybutyrate (PHB) is natural thermoplastic polyester which is produced by bacterial fermentation and degrades fully in the environment without forming any toxic products [10].

Many studies have therefore being focussed on the PHB producing micro organisms under the conditions of nutrient stress especially marine bacteria have strategies to produce this type of novel compounds due to the unique environment they survive.

S.No	Name of the Test	KMM1
1	Indole Production Test	-ve
2	Methyl Red Test	-ve
3	VogesProskauer Test	+ve
4	Citrate utilisation Test	-ve
5	Oxidase Test	+ve
6	Catalase Test	+ve
7	Urease Test	+ve
8	Carbohydrate	+ve
9	Fermentation Test	-ve
10	Oxidative Fermentation	-ve
11	Nitrate reduction Test	+ve

Table-1: Biochemical properties of KMM1

In the present work, from marine samples, KMM1, KMM2 and KMM3 were isolated. Among them **KMM1**was identified positive for presence of lipophilic PHB granules. PHB granules were observed as dark blue-black droplets [Figures -1,2] in the pink vegetative cells [11] by using Sudan black B staining method.

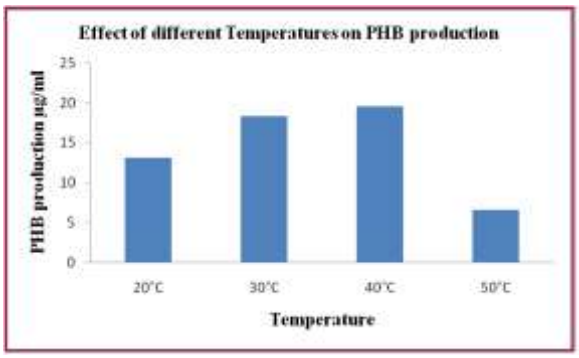

Figure-9: Effect of temperature on PHB production

KMM1 was found to be gram positive, rod shaped and non motile under microscope. The colonies are small, circular, raised and dull white in colour with wavy margin. The bacterial strain was typically positive for Voges Proskauer, Nitrate Reduction, Carbohydrate fermentation, Urease test and Catalase tests and showed negative reaction for Indole, Methyl Red, and Oxidative fermentation tests (Table-1).

Amplification of 16s r RNA and other genotypic approaches are taking over traditional ways for identification of genus to the species level. Among the used methodologies PCR is widely practiced. The gene 16s rRNA is the tool mainly used for molecular identification of bacteria. The NCBI BLAST search program showed that the sequence data of KMM1 had 99% identity with *Bacillus amyloliquefaciens* 16s r RNA sequencing and phylogenetic tree was constructed [12]. KM091730 is the accession number allotted by Genbank for the submitted nucleotide sequence.

The fermentation medium was optimized by testing various carbon sources, nitrogen sources, NaCl concentration, pH and temperature. The maximum yield of the product 19.6µg/ml was obtained after optimizing the fermentation medium. The production of polyhydroxybutyrate at various stages of optimization is given in the following figures 3 to 9. Results from the study indicate that Glucose at 2.5%, yeast extract at 0.2%, C:N ratio at 2.5:0.2, neutral pH and 40^0 C temperature are optimal conditions for fermentative production of PHB that it gave a yield of 19.6µg/ml.

4.0 Conclusion

Among the 3 isolates, PHB producing KMM1 was identified positive for Voges Proskauer, urease, Oxidase, Catalase and Carbohydrate fermentation tests. KM091730 is the accession number allotted by Genbank for the submitted nucleotide sequence and identified as *Bacillus amyloliquefaciens*. Glucose (2.5%), yeast extract(0.2%), C:N ratio(2.5:0.2), neutral pH and temperature of 40^0 C are found to be optimal for r fermentative production of PHB.

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