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Preliminary optimization of PHB production by *Vibrio* sp. MCCB 237 isolated from Marine Environment

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Abstract

Polyhydroxybutyrate (PHB) is the most widely studied polyester of microbial origin and is the representative of a group of such polyesters known as polyhydroxyalkanoates (PHA). PHAs are polymers that are synthesized by microorganisms under imbalanced growth conditions and serve as carbon and energy reserve. The material properties of PHAs are similar to various petrochemically-derived thermoplastics and elastomers that have immense use in our daily life and hence are considered as possible substitutes for synthetic, non-degradable polymers. The commercial exploitation of these polymers is still restricted by the high cost of production incurred. The success in the biodegradable plastic strategy largely depends on the isolation of potent PHB-producing bacteria and optimizing culture parameters for maximum PHB biosynthesis. In the present study the efficacy of various carbon and nitrogen sources were investigated for enhanced PHB production using Vibrio sp. MCCB 237 isolated from marine environment, which provides a meagerly exploited resource for possible biopolymer-producing novel bacteria. Quantification of PHB was done spectrophotometrically and characterized using FTIR. Among the tested carbon sources pectin followed by glycerol gave the highest yields and among the nitrogen sources, yeast extract yielded better quantity of PHB. These results are being implemented in further optimization of culture parameters.

Keywords: Biopolymers, polyhydroxyalkanoates, polyhydroxybutyrate, degradable, bioplastics.

Introduction

Polyhydroxybutyrate (PHB) was first discovered by Lemoigne in 1926 in *Bacillus megaterium*[1]. Since then a large diversity of PHAs have been discovered and the numbers are still growing[2]. Polyhydroxybutyrate is the representative of a group of compounds called the polyhydroxyalkanoates or PHAs which are optically active, thermoplastic, microbial storage polymers/polyesters accumulated in response to unbalanced growth conditions[3,4]. PHB is the most abundant and common type of polyhydroxyalkanoate, which is often used as a taxonomic characteristic. PHAs have a sufficiently high molecular mass which infers on them characteristics similar to conventional, petrochemically-derived plastics and hence are considered as possible substitutes for synthetic plastics. An added advantage of PHAs is that not only is their production based on renewable resources, they are also biodegradable. The thermoplastic properties of PHAs have enabled their varied applications in packaging and coating, hot-melt adhesives, personal hygiene products etc[5]. Initial interest in bacterial polyesters was spurred on by price hike of crude oil in the 1970s. Later on environmental concerns like plastic waste accumulation have also persuaded the attention towards production of biodegradable polymers from renewable sources as they have low environmental footprint[6]. The past few decades have seen tremendous increase in the knowledge base of biodegradable polyesters of microbial origin. Though more than 300 microorganisms are known to synthesize PHAs, very few like *Alcaligenes latus, Alcaligenes eutrophus, Azotobacter vinelandii, Pseudomonas oleovorans, and recombinant Escherichia coli* are used for PHB production on an industrial scale[2,7,8]. The commercial exploitation of these polymers is still restricted by the high cost of production incurred. The economic production of these biodegradable plastics relies on high productivity. The major contributing factors in the cost of PHA synthesis are the carbon source and the recovery process. Considerable effort has been directed towards exploring cultivation strategies involving isolation of efficient bacterial strains, use of inexpensive and renewable carbon substrates, more efficient fermentation and recovery methods, etc., to overcome this limitation.

The low price of crude carbon substrates such as cane and beet molasses, cheese whey, plant oils and hydrolysates of starch, cellulose and hemicellulose make them attractive substrates for producing PHAs by several bacteria utilizing them[2,7,9]. Use of renewable waste substrates for polymer production is an environment-friendly option which on one side contributes to the reduction of waste accumulation while on the other provides value-addition of by-products. Such bioconversions would directly benefit the environment by obtaining biodegradable polymers[10]. Optimization of culture conditions and media composition contributes significantly towards enhancement of the desired product and thereby making the fermentation process more cost effective[11]. Studies have shown that novel polymers can be produced in low-cost, high productivity

fermentations through manipulations of metabolic pathway for PHA production[12].

In the present study, as a preliminary screening before further optimization studies, various carbon and nitrogen sources were investigated for higher yield of PHB using bacteria isolated from marine environment. An advantage of using marine microorganisms for PHB production rests on the fact that these can be propagated in simple media like ZoBell's where the basal medium is sea water, rich in various mineral salts. This in turn negates the need of additional minerals to be added to the medium.

Material and Methods

Microorganism: The PHB producing bacterium used in the present study was isolated from decaying marine algae *Ulva faciata* off Thiruvananthapuram, India. Around 10 g of algal sample was macerated and serially diluted in sterile seawater and plated onto ZoBell's Marine agar plates[13], and incubated at 28°C for 24 hours. Single colonies were picked and purified by repeated sub-culturing on ZoBell's agar plates. Purified isolates were screened for PHB production by nile red staining following a previously described method by Ostle and Holt with slight modifications[14]. The most intensely fluorescent isolate following staining was selected and was found similar to *Vibrio* sp. MCCB 237 by 16 S rRNA sequence analysis. The isolates were maintained at 4°C on ZoBell's Marine agar slants and subcultured every month.

Media and culture conditions: In prior experiments, optimum temperature and pH were determined for the growth of the bacteria by incubation in ZoBell's broth under different ranges of temperature, pH and agitation rates, following which a temperature of 30°C, pH of 7.5 and agitation of 125 rpm were selected for further screening of carbon and nitrogen sources.

Effects of Carbon source on PHB production: The effect of different carbon sources on PHB production was studied by incubating the bacterial isolate for 72 hours by replacing the peptone in ZoBell's medium with different carbon sources, having the following composition: carbon source 0.5 %, $FePO_4$ 0.01 % in aged sea water of 30 ppt salinity and supplemented with yeast extract 0.1 % for nitrogen and growth factors. The carbon sources used were glucose, glycerol, sodium malonate, sucrose, starch, pectin, lactose, xylose, and succinic acid. The selected carbon source was used for the next stage of optimization.

Effects of Nitrogen source on PHB production: Different nitrogen sources were tried to study their effect on PHB yield. The nitrogen sources used were ammonium dihydrogen orthophosphate, ammonium chloride, ammonium nitrate, potassium nitrate, ammonium ferrous sulphate, ammonium sulphate and yeast extract. The nitrogen source was supplied at 0.1 % in the medium containing 0.5 % v/v of glycerol, 0.01% $FePO_4$ in natural aged sea water of 30 ppt salinity. Cultures were incubated for 72 hours.

Analytical Methods: Dry cell weight was obtained gravimetrically after centrifugation followed by washing in distilled water and drying of pellets at 90°C till constant weight was reached. PHB content was estimated spectrophotometrically. Cell pellet obtained by centrifugation at 4°C, 10000 rpm for 10 minutes was suspended in equal volume of 4% sodium hypochlorite for incubation at 37°C for 1 hour. The mixture was centrifuged after incubation and washed sequentially with water, acetone and alcohol. The residue was then refluxed with chloroform at 60°C for 1 hour. Chloroform extract was filtered and evaporated to dryness. Determination of PHB yield was performed by conversion to crotonic acid by treatment with conc. H_2SO_4 and absorbance measured at 235 nm[15]. Ultraviolet absorption spectrum of the polymer was analyzed between 200 and 400 nm with UV spectrophotometer (Systronics, India).

Characterization: Polymer sample was characterized using Perkin Elmer (Model – Spectrum 100) FTIR spectrophotometer. The spectra were recorded in the range from 4000 to 600 cm^{-1}, using attenuated total reflectance (ATR).

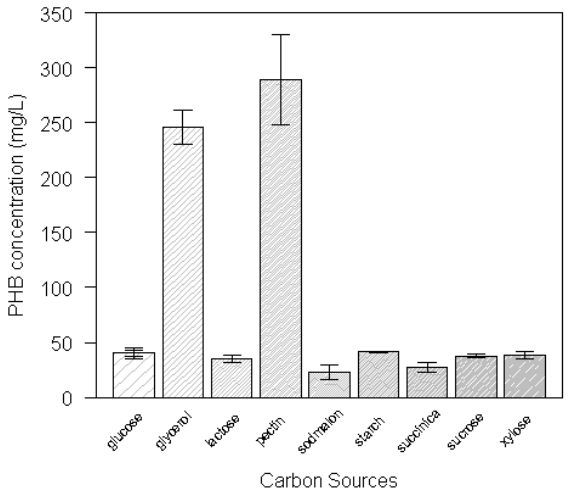

Figure-1
Effect of different carbon sources on PHB production by *Vibrio* sp. MCCB 237, Error bars represent standard deviations

Results and Discussion

Batch experiments were performed in shake flasks for production of PHB. The flasks were incubated at 30°C for 72 hours at 125 rpm. Preliminary experiments proved that processing parameters are crucial in the enhancement of PHB production. As is evident from figure 1, *Vibrio* sp. MCCB 237 used in this study is a versatile bacterium that can utilize various carbon sources for PHB production to varying degrees. Among the carbon sources tested for their efficacy, pectin 0.5% w/v and

glycerol at 0.5% v/v yielded PHB at about 18 and 15 % respectively of the total biomass. The biomass and PHB content from the different carbon sources are summarized in table 1. There are no reports on the use of pure pectin, commercial or otherwise, as carbon source aiming PHA production, though pectin hydrolysates have been used by Locatelli *et al*., for PHA production from *Cupriavidus necator*[16]. Pectins are structural polysaccharides consisting mainly of galacturonic acid and neutral sugars such as rhamnose, galactose, arabinose and xylose. Upon degradation of pectin, the reducing groups released into the media might lead to excess of available carbon which could account for the higher quantity of PHB produced. When dry powder form of pectin is added to water it hydrates very rapidly forming clumps. Moreover, the presence of calcium ions (a significant component of sea water) also influences the gelling property of pectin[17]. At higher concentrations this property could lead to the formation of a heterogeneous media solution resulting in inefficient utilization of pectin by the bacteria and thereby wastage of carbon source. From industrial point of view, an additional step of pectin hydrolysis (acid hydrolysis or enzymatic hydrolysis) for a homogeneous culture medium before fermentation would lead to increase in the cost of production. In this context glycerol is a good alternative which is the structural component of various lipids. Glycerol is also a compatible solute[18]. Earlier works have reported the use of glycerol by *Ralstonia eutropha, Methylobacterium rhodesianum*, and *Pseudomonas oleovorans*[19,20]. Glycerol is considered to be a very energetically favorable substrate for the formation of acetyl-CoA which is the precursor for PHB synthesis. It is the principal by-product obtained from soap industry and also a co-product of the biodiesel industry. Crude glycerol from biodiesel industry has been used without further purification which makes the use of glycerol even more attractive[12].

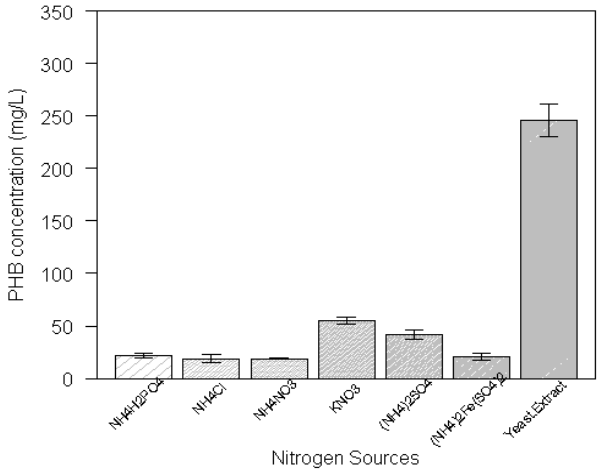

Figure-2
Effect of different nitrogen sources on PHB production by *Vibrio* sp. MCCB 237, Error bars represent standard deviations

Table-1
PHB production by *Vibrio* sp. MCCB 237 using different carbon sources

Carbon Source	Cell Dry Weight g/L	PHB g/L	PHB Content (%)
Glucose	0.9	0.043	4.7
Glycerol	1.68	0.246	14.64
Lactose	1.71	0.035	2.05
Pectin	1.58	0.289	18.29
Sodium Malonate	1.11	0.023	2.07
Starch	1.06	0.041	3.87
Succinic acid	1.42	0.027	1.90
Sucrose	1.38	0.038	2.75

Among the nitrogen sources, yeast extract resulted in the highest yield of PHB in the medium containing 0.5 % v/v of glycerol. Yeast extract is a complex nutrient composed of many amino acids, sugars, vitamin B, minerals etc., and is known to be an excellent stimulator of bacterial growth. The PHB yield and biomass obtained using *Vibrio* sp. MCCB 237 from various nitrogen sources is summarized in table 2. All other nitrogen sources yielded good biomass but relatively low PHB content.

Table-2
PHB production by *Vibrio* sp. MCCB 237 using different nitrogen sources

Nitrogen Source	Cell Dry Weigt g/L	PHB g/L	PHB Content (%)
Ammonium ferric sulphate	2.51	0.0206	0.8
Ammonium sulphate	2.1	0.042	0.2
Potassium nitrate	2.82	0.055	1.9
Ammonium chloride	2.75	0.0188	0.6
Ammonium dihydrogen orthophosphate	2.8	0.0214	0.76
Ammonium nitrate	2.4	0.0189	0.78
Yeast extract	1.68	0.246	14.64

The FTIR spectroscopy of the extracted polymer (data not shown) showed an intense band at 1720 cm^{-1} corresponding to the ester linkages of PHB and at 1275 cm^{-1} corresponds to the –CH group which are characteristics of PHB. The band around 1379 cm^{-1} ($–CH_3$) is insensitive to the degree of crystallinity and that around 1183 cm^{-1} (-C-O-C-) is sensitive to the amorphous state. The ratio of these band intensities is a measure of the crystallinity of the polymer, known as crystallinity index or CI[21].

Conclusion

The present study has revealed that *Vibrio* sp. MCCB 237, selected for the production of PHB is a versatile organism capable of utilizing various carbon and nitrogen sources for growth and PHB production. The fast growth and ease of cultivation can lead to better PHB yields in shorter time period. Utilization of carbon sources like pectin and glycerol is an added advantage because it points towards the possibility of commercial production of value added products such as PHB from agricultural and industrial wastes which will not only ensure reduction in production costs, but will also go a long way in reducing the environmental hazards caused due to waste accumulation. Among the carbon sources investigated, glycerol and among nitrogen sources, yeast extract emerged as the feasible candidates for further scale-up studies. Further optimization of culture parameters by *Vibrio* sp. MCCB 237 is under progress.

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