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Isolation and characterization of indigenous *Ralstonia* strain, YRF1 for high Polyhydroxy Alkanoates (PHA) production

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ABSTRACT

The bacterial polyesters may be considered as "Green Plastics", because of their biodegradable nature. These polyesters can be employed for packaging and coating materials / as biodegradable carriers and applied in biomedical field. PHA producing Bacteria were isolated from the soil samples collected from various locations of Salem district in Tamil Nadu, India. The bacterial consortium was screened and confirmed for indigenous *Ralstonia* strains producing Polyhydroxy alkanoates (PHA) by colony morphology and biochemical analysis. The PHA production was confirmed by the presence of lipid cellular inclusions using Sudan Black B staining. Three strains (YRF2a, YTF2 and YRF1) among the 38 isolates tested were found to be *Ralstonia* strains. The positive isolates were then cultured in E2 broth containing 2% glucose (w/v) to study the PHA production. The correlation between PHA production and cell dry weight were studied and found to be statistically significant. Among the three isolates tested the strain YRF1 showed highest PHA production and peaked in degradation also. The high PHA yielding strain (YRF1) was further characterized for PHA production in large quantity with different physical and chemical parameters *viz;* pH, incubation temperature, carbon sources, concentration of nitrogen, incubation period. The result presumed that at pH 7, at 37°C and 72 hours of incubation period accompanying with glucose as carbon source and reducing nitrogen concentration to 1/6 in growth medium increased the accumulation of PHA by 88%, a highly feasible amount. The extracted PHA was apparently confirmed by FT-IR and NMR analysis.

Introduction

Polymers originating from living organism are termed biopolymers, which are different from manmade synthetic polymers such as PVC and polypropylene. PHAs are microbial polyesters, synthesized by numerous microorganisms having dual function as a reserve compound and as a stress metabolite accumulating in response to stress condition (Anupama *et al.,* 2010). Problems regarding global environment and solid waste management have created much interest in the field of biodegradable plastics in current years.

Many types of biodegradable plastics are available; among them Polyhydroxy alkanoates (PHAs) are the only 100% biodegradable polymers possess properties similar to various synthetic thermoplastic like polypropylene making them useful for a wide range of applications.

PHAs are synthesized by numerous bacteria as intra cellular carbon or as energy storage compounds and are accumulated as granules in the cytoplasm of the cell (Shamala *et al.,* 2003). Stiffness and brittleness which are the properties of a normal plastic are also shown by bacterial polymers (Amutha *et al.,* 2010) and therefore can be used in variety of application like the manufacturing of bottles, plates (Oliveria *et al.,* 2004). Renewable carbon sources can be used for the production of these water insoluble strong polymers. The production cost of bioplastics being the main criteria can be reduced by using cheap carbon sources and nutritional supplements with feeding strategies (Mazur *et al.,* 2009). The bacterium *Ralstonia eutropha* produces has high PHA productivity in a short period (Chanprateep *et al.,* 2008). The main aim of our study is to isolate high yielding indigenous *Ralstonia* strain and to optimize the production parameters which will result in removal of the bottlenecks faced in bioplastic production and research.

Materials and Methods

Isolation of PHA Producing *Ralstonia* Strain

41 soil samples (10g each) were collected from different environmental conditions and locations of Salem district, TamilNadu, India by scraping off the soil surface with sterile spatula and collected the soil sample from a depth of 2-5cm and at a distance of about at least 1Km each from different environment conditions. All samples were kept aseptically in sterile plastic bags and stored at 4°C for the isolation of bacterial strain with prospective of PHA production. Bacterial strains from soil samples were isolated by serial dilution and spread plate method. Isolated bacteria were characterized based on colony & cellular morphology and biochemical analysis. The presence of PHA as intracellular granules was confirmed by Sudan Black B staining from 48 hours grown strains in E2 broth by examining under the microscope at 40X and 100X oil immersion objective.

Extraction, Quantification of PHA and Optimization of Growth Parameters

The indigenous *Ralstonia* isolates YRF2a,YTF2,YRF1 were grown in 250 ml erylenmeyer flasks containing 50 ml E2 mineral medium incubated at 37^0C for 48 hrs on rotary shaker at 150 rpm and the process was triplicated. Sodium hypochlorite digestion method for PHA extraction from purified indigenous *Ralstonia* strains was followed and dry cell weight

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(DCW) was determined (Joshi *et al.*, 2010). Percentage of PHA was calculated as follows:

Percentage of PHA = (weight of PHA / dry cell weight) × 100.

The correlation between the production of PHA and DCW of the isolates was determined by Spearman's correlation coefficient. The level of significance at 5% was also calculated. Highest PHA yielding indigenous *Ralstonia* strain (YRF1) was subjected to different physical and chemical production parameters (Table 1) for optimization.

Degradation of Extracted PHA by Soil Microbes

The nitrogen free mineral agar medium (g/l) was used for degradation studies (glucose-20g, K_2HPO_4-0.8g, KH_2PO_4-0.2g, $MgSO_4.7H_20$-0.5g, $FeCl_3.6H_2O$-0.025g, $Na_2MoO_4.2H_2O$-0.005g, $CaCl_2$-0.05g and agar-15g, pH 6.9). After sterilization, a portion of the medium was poured in a sterilized petridish and allowed to solidify. Another portion of the medium melted and cooled to 45-50°C and a sufficient amount of sterile PHA granules suspension was added to give final concentration of 2.5% (w/v) in the medium (Granule- agar suspension) was poured over the surface of solidified medium to form a thin layer. Then, serially diluted samples were spread over the medium and incubated for 3-5 days at 37°C.

Biopolymer Analysis

The biopolymer extracted by sodium hypochlorite method was analyzed by FT-IR spectroscopy (model Spectrum RXI). In FT-IR analysis the extracted biopolymer was used under the spectral range of 4000-400cm^{-1} to confirm the functional group. 1H NMR spectra was acquired by dissolving the polymer in deuterochloroform ($CDCl_3$) at a concentration of 32 mg/mL and analyzed on AV 300 spectrometer at 300K with 9.65 ms pulse width, 2 Sec pulse repetition, 6172.8 Hz spectral width and 32,768 data points. Tetramethylsilane was used as an internal shift standard.

Result

Isolation and Screening of PHA Producing *Ralstonia* Strain

Among 41 soil samples collected from fertile land, waste land and hilly regions; 3 indigenous strains (YRF2a, YTF2 and YRF1) from 38 bacterial isolates were confirmed as *Ralstonia* strain, based on their colony and cellular morphology as well as biochemical characterization (Table -2). PHA content of the strains was confirmed using Sudan Black-B staining. PHA granules appeared as blue black droplet (Sudanophilic nature) and cytoplasmic area appeared as pink colour under 100x oil immersion light microscope (Figure -1).

Figure 1: Sudan black staining

Production, Extraction and Quantification of PHA

Isolated indigenous *Ralstonia* strains (YRF2a, YTF2 and YRF1) produced PHA under normal condition in E2 medium. The results are statistically significant. The YRF1 strain showed maximum PHA production. Its production was further optimized using different parameters (pH, temperature, incubation period, carbon sources and nitrogen concentration (Table-3).

Degradation of PHA by Soil Microbes

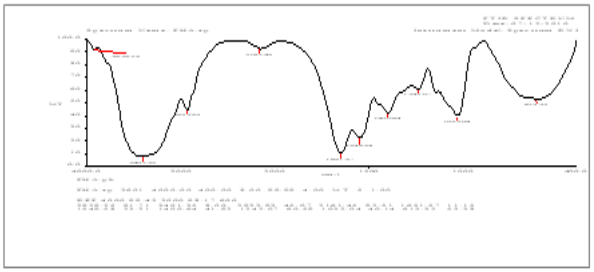
Extracted PHA 2.5% (w/v) was utilized as carbon source by soil borne microorganisms on nitrogen free mineral agar medium. Clear zone was observed that indicated that extracted PHA is readily degradable biopolymer (Figure-2).

Figure 2: Degradation study

Biopolymer Analysis

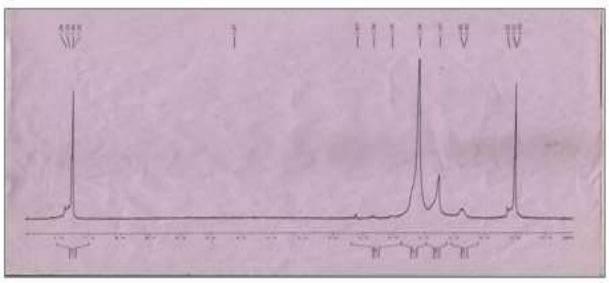
FTIR analysis showed the functional group of extracted PHA as C=O. Purified PHA was characterized with respect to FT-IR analysis. The spectra of the isolated polymer revealed that the absorption band was corresponding to the ester carbonyl group, alkyl group, carboxylic acid and chemically it is a hydroxyester in nature. The absorption bands are assigned to -CH2- vibrations and as a conformational -CH2- group of the helical chains (Figure 3).

Figure 3: FT-IR Analysis of PHA Extract



In NMR analysis, major peaks at various ppm's viz; 0.02, 1.58 and 7.28 were due to the resonance absorption that indicates the existing PHB structure (Figure 4).

Figure 4: NMR Analysis of PHA Extract



Discussion

Polyhydroxy alkanoates (PHA) are storage polymers accumulated by a variety of bacteria under condition of nutrient limitation and carbon excess. Once PHAs were extracted from the bacterial cells, they show material properties similar to some common plastic such as polypropylene. Due to its bacterial origin, many microorganisms evolved the ability to degrade these macromolecules (Lara *et al.*, 1999). However soil has rich biodiversity of microbial population including PHA producing

organisms. From the 38 isolates tested, 11- gram positive rod shaped bacterium was identified based on cellular morphology.

Based on the colony morphology and biochemical analysis, the three indigenous bacterial isolates YRF2a, YTF2, YRF1 were confirmed as *Ralstonia* strains. Sudanophilic nature of the PHA granules was observed (Amutha *et al.,* 2010 and Attia Razzaq *et al.,* 2010).

In this study, production parameter optimization results gave the pH range, incubation temperature, carbon sources and nitrogen concentration that increase the yield. Prolonged incubation under nutrient limiting conditions cannot be carried out because, stored PHB is utilized as energy in presence of depolymerase (Joshi *et al.,* 2010 and Sayyed *et al.,* 2009). Carbon sources in the form of medium chain length, monosaccharides and ammonium sulphate as nitrogen source are easily utilized by the microbes rather than unrelated sources (glycerol, citrate) (Joshi *et al.,* 2010 and Chandrashekharaiah *et al.,* 2008).

Studies on carbon sources with different carbon sources such as glucose sucrose, fructose, lactose and n- octane in nitrogen free medium with *Alcaligenes eutropus* (R. *eutropha*) gave PHA concentration of 4.14g/l with glucose than other carbon sources (Amutha *et al.,* 2010) Our study showed the maximum accumulation of PHA at pH 7 than at pH <6. PHA accumulation was reported to increase with an increase in glucose concentration till a threshold level than it either remained steady or declined. Decreasing the nitrogen concentration at a certain uniform rate increased the PHA accumulation till a threshold level. Their result indicated that at 30°C, maximum CDW and P (3HB) biopolymer were 2.6g and 1.63g/l respectively. Results from this study contributed significantly to our objective of seeking indigenous bacteria which could synthesize high percentage, stable and biodegradable PHA, when incubated at 27°C and 37°C yielded 23% and 51% respectively. 72 hours incubation period was found optimum for the selected *Ralstonia* strains. Other studies by Sharifzadeh Baei *et al.,* 2009 reported the maximum accumulation after 72 hours at 30°C. 48 hours incubation period for PHA accumulation, when its production was carried out in nitrogen limited culture medium.

The degradation of P (3HB) studied was concomitant with a drastic increase in polydispersity. This implies that scission of the P (3HB) polymer chain proceeded heterogeneously. However, random scission (endo-type degradation) was also observed (Chen *et al.,* 2006).

FTIR analysis showed the functional group of extracted PHA as C=O as same was reported by Amutha *et al.,* 2010. In NMR analysis, the peak values at various ppm viz 0.02, 0.88, 1.25, 1.58 and 7.28 indicates the existing PHB structure. Major peaks at 1.2, 2.5 and 5.2 ppm were due to the resonance absorption of methyl (CH3), methylene (CH2) and methane (CH) groups, respectively, in 3- hydroxybutyrate (3-HB) units. Two additional peaks at 0.9 and 1.6 ppm appeared due to presence of 3-hydroxyvalerate (3-HV) units (Chen *et al.,* 2006). Therefore the FT-IR result formed with *R. eutropha* is in complete agreement with the earlier reports of Rohini *et al.,* 2008 and Valappil *et al.,* 2007.

Further, extracted PHA subjected in microbial growth as carbon source, on nitrogen free mineral agar medium. The zones formed around the colonies indicated the utilization of PHA as a carbon source. As a result, the extracted PHA was concluded as biodegradable. *R. eutropha* was the production organism of choice for ICI in the development of commercial production facilities for P (3HB-3HV). This grows well in minimal medium at 30 °C on a multitude of carbon sources. The combination of developments in metabolic engineering of amino acids and PHA pathways provides a tremendous benefit for the successful generation of economic P (3HB-3HV) producers. It is therefore expected that other biotechnological processes will aid in the production of some specific PHAs as well (Zazali Alias *et al.,* 2005).

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Conflict of Interest

All the authors of this article do not have any conflict of interest regarding the authorship of this research article.

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Table 1: Optimization of growth parameters for PHA production

S. No	Parameters	Range	Incubation condition
1	pH	5, 6, 7, 8, 9	37°C , for 72 hrs
2.	Temperature	27°C & 37°C	27°C,37°C for 72 hrs
3.	Incubation period	(24,48,72,96,120,144) hrs	37°C each
4.	Carbon source	Glucose, fructose, sucrose, maltose and starch soluble	37°C , 72 hrs each
5.	Nitrogen concentration	1/2 ,1/4,1/6,1/8,1/10	37°C , 72 hrs each

Table 2: Colony, Cellular morphology and Biochemical characterization of indigenous *Ralstonia* strain

Colony Morphology		Biochemical Characterization	
Test	Result	Test	Result
Configuration	Circular	NaCl treatment	+ve
Elevation	Low convex	Catalase Test	+ve
Pigment	-	Citrate Utilization Test	+ve
Opacity	Transparent	Nitrate Reduction Test	+ve
Texture	Moist	Methyl Red Test	_ve
Cellular Morphology		Voges Proskauer Test	_ve
Gram Staining	+ve	Sugar Fermentation Test (glucose, fructose, lactose, maltose, sucrose)	+ve
Cell shape	Rods		
Arrangement	Groups		
Motility	+ve		

Table 3: Optimization of Growth Parameters for *Ralstonia eutropa* strain YRF1

Temp. (°C)	PHA Yield (%)	pH	PHA Yield (%)	Carbon source	PHA Yield (%)	N_2 source concentration	PHA Yield (%)
27	22.75±0.56	5	20.40±0.53	Glucose	51.30±0.55	1/2	50.00±0.62
		6	40.03±0.89	Sucrose	32.00±0.43	1/4	41.93±0.73
37	51.30±0.55	7	51.30±0.55	Starch	No yield	1/6	88.00±0.45
		8	33.15±0.07	Maltose	38.35±0.26	1/8	17.24±0.64
		9	50.05±0.34	Fructose	34.80±0.36	1/10	20.83±0.55