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FULL LENGTH ARTICLE

Microbial Production of PHA Using Waste Marigold Flowers

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ABSTRACT
Although synthetic plastics are used as a main commodity in day to day life of human being, it has many adverse effects on environment as it is not biodegradable and accumulation of the waste plastics affects the ecosystems. A better alternative to the synthetic platics are the bio degradable plastics. Polyhydroxyalkonates (PHA) are one of the biodegradable plastics which are produced by different micro- organisms. These micro- organisms accumulate PHA in the form of granules near cell wall under stress conditions. But, the production of PHA is expensive due to requirement of costly carbon source. Therefore attempts are being done to reduce the production cost by replacing the costly substrate by cheaper sources. Here in this study, waste Mariegold flowers are used for the production of PHA. Smf and SSF both the methods have been used for the production, where the SSF showed better accumulation of PHA. The production was optimized for SSF and futher extraction and biofilm formation was done to get the PHA polymer. The production was confirmed with FTIR.
Keywords*: Polyhydroxyalkonate (PHA), Solidstate Fermentation (SSF), Biofilm, Waste mariegold flower, Ralstoniaeutropha*

INTRODUCTION

Synthetic plastics are one of the greatest inventions of mankind and have been developed into a major industry and indispensable commodity in human's life. They are designed in a way to suit the constant performance and trustable qualities that are used for long life span, therefore causing them to be inert to natural and chemical breakdown. The durability of these disposed plastics contributes to the environmental problems when they go into the waste stream. It was predicted that an increase of 25 million tonnes of synthetic plastics accounted to 230 million tonnes from year 2006 to 2009[1]. As the natural environment is continuously polluted by these harzardous plastics, the development and production of environmental-conserved biodegradable plastics are rapidly expanding in order to trim down our reliance on synthetic plastics. Bio-based materials such as polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyanhydrides, polyisoprenoids and polyphenols are potential candidates for substitution of synthetic plastics. Among these, polyhydroxyalkanoate (PHA), which belongs to the group of polyoxoesters has received intensive attention because it posseses biodegradable thermoplastic properties. PHA is a family of naturally occurring bio polyester synthesized by various microorganisms. Some bacteria have been reported capable to produce PHA as much as 90% (w/w) of dry cells during depletion of essential nutrients such as nitrogen, phosphorus or magnesium[2][3]. Production of PHA is challenging due to expensive raw materials and it is estimated up to 20-50% of the total production cost. Thus reducing the higher PHA production cost is desirableusing cheaper raw materials [4]. One possible strategy for reducing costs is to utilize the alternative substrates. Many cheaper substrates have been reported. In this study, waste mariegold flower has been used as a cheaper substrate for the production of the PHA. The production of PHA was done with both SSF and SmF.

MATERIALS & METHODS:

A. Micro organism:

Extensive literature review was performed and the *Ralstonia eutropha*(MTCC 1472)[3][5][6]was selected as PHA producer since, it has the ability to accumulate a large quantity of PHA under unbalanced growth conditions, it can grow on various substrates. It was procured from the Department of Biotechnology, SIESGST, Nerul, Mumbai, India. *R. eutropha* was maintained on nutrient media 3 agar slants at 4˚C.

B. Substrates and Chemicals:

Waste mariegold flowers were procured from nearby temples. And the other reagents like Nutrient Agar 3 medium, Sudan Black, Methanol, Sodium hypochlorite were obtained from HiMedia.

C. Pretreatment of Substrate

Flowers were tested for PHA production in different forms. Among them one form is the flower in powdered form as substrate. Powdered form of flowers is prepared from waste marigold flowers by twice washing them in appropriate amount of distilled water. It was then dried in Hot air Oven at 80^{o}C for overnight and powdered by using grinder. The powder obtained was then screened through mesh of 60 and then used as a substrate for the study. The substrate was sterilized by two methods: autoclave sterilization and ethanol sterilization.

D. Polyhydroxyalkanoate production in nitrogen deficient media using glucose as a standard carbon source :

Since PHA is produced under nutrient limiting conditions, nitrogen deficient media was chosen and supplemented with novel c-sources like whole flower, shredded flowers and the powdered form in place of glucose. The composition of the nitrogen deficient media is as follows: Composition (g/l): Glucose 15.000, Magnesium sulphate 0.200, Sodium chloride 0.100, Potassium di-hydrogen phosphate 0.500, Peptone 2.500, Yeast extract 2.500. The production flask was inoculated with 10% inoculum comprising *R. eutropha*. The flask was incubated at 30˚C for 24-48 hrs [6][7][8]

E. Submerged state fermentation:

Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. SmF technique can be utilized for the production of PHA. Here all the contents were weighed and added in 500 ml conical flask to prepare 250 ml of production media and 250 ml of distilled water was added to it. The pH was adjusted in the range of 6.5 to 7.1 and the media was then autoclaved at 121^{o}C for 20 minutes. The media was inoculated with *Ralstonia eutropha* and incubated for 48 hours at 30^{o}C at 120 rpm [10][11][12][13].

F. Solid state fermentation

Solid-state fermentation (SSF) is defined as the fermentation process in which microorganisms grow on solid materials in the absence of free water. Here all the contents were weighed and added in 250 ml conical flask to prepare 250 ml of production media. The pH was adjusted in the range of 6.5 to 7.1 and the media was then autoclaved at 121^{o}C for 20 minutes. The media was inoculated with *Ralstonia eutropha*. 15 ml of distilled water was added as initial moisture content and the flask was incubated for 48 hours at 30^{o}C. Glucose was used as a standard carbon source for comparing the results [7][8][9].

The production was optimized for carbon source, incubation time, initial level of moisture content, inoculum level and temperature.

G. Extraction of PHA

Microbial PHA is stored as insoluble intracellular granules. Methods to recover PHA would typically involve cell wall/cell membrane lysis, solubilization and purification of PHA component, and precipitation of PHA polymer [3,16]. The extraction method of PHA was standardized for the novel c-sources. For SSF, after incubation distilled water was added and stirred vigorously at various intervals i.e. half hour, one hour and one and half hour. The biomass was then obtained by filtering the fermented broth and centrifuging the filtrate. The biomass containing intracellular PHA was collected by centrifugation at 8000 rpm for 15 min for SmF. The dry weight of the residual biomass was estimated and then the cells were treated with 6% sodium hypochlorite solution. The resulting mixture was incubated at 30˚C for 60 min. After incubation the mixture was collected in centrifuge tubes and treated with chloroform. The resulting biphasic mixture was centrifuged and the clear sodium hypochlorite layer was discarded. The chloroform layer was collected and the PHA was precipitated using chilled methanol. The dry weight of the extracted PHA was determined [27].

H. Analytical Methods:Qualitative method (Screening for production of PHA)

Using Sudan Black B screening method the microorganisms were screened for accumulation of PHA as energy storage. In this, the cells were heat fixed and stained with Sudan Black B in order to stain the PHA granules. The cells were then counterstained using safranin dye and observed under oil immersion lens of light microscope. Blue black granules of PHA were observed against pink cytoplasm of cells.

I. Quantitative methods

PHA yield was estimated by two methods: (1) Cell dry weight analysis method (2) Crotonic acid assay

1. Cell dry weight analysis method [12,13,14]

In order to measure the cell dry weight, 80 ml samples were withdrawn and centrifuged at 8000 rpm for 15 min. The supernatant was removed and the pellet was re-washed by re-suspending in an equal amount of distilled water and repeating the centrifugation process. The washed cells were poured on a pre-weighed petri plates and dried in an oven at 80oC, and allowed to cool until constant weight was obtained. The final weight was thus recorded. The biomass weight (cell dry weight) was determined by

subtracting the initial weight of the petri plate from the final weight. The %PHA accumulation was determined by using the following formula:

$$PHA\ Accumulation\,(\%) = \frac{Dry\ weight\ of\ extracted\ PHA\ \left(\frac{g}{l}\right)\ X\ 100}{Dry\ Cell\ Weight\ \left(\frac{g}{l}\right)}$$

2. Crotonic acid assay [CAA][8, 24]

The extracted PHA was treated with concentrated sulfuric acid and incubated at 100˚C for 10 min. The resulting solution was estimated using UV-VIS spectrophotometry against concentrated sulfuric acid as blank at 230 nm. The resulting concentration of PHA in the sample was determined using the standard graph of concentration of crotonic acid against its O.D value. The standard crotonic acid solution was prepared by dissolving 400 µg of crotonic acid in 100 ml of concentrated H_2SO_4 and the concentration of standard solutions of crotonic acid was varied.

J. Biofilm formation [26]

Mixture of starch powder and PHA in certain ratio (15:1) and8 mL of acetic acid was added to 105 mL of water in a 250 mL beaker. 10 mL of glycerin was added to the water solution and the contents were stirred till the solution appeared opaque. The 250 mL beaker of the starch solution was placed on a hot plate set at 130°C and continued to stir until the mixture becomes thick and almost transparent (about 5-7min). In a separate 50 mL beaker 2.5 g of sodium bicarbonate was added to 30 mL of water. The solution of sodium bicarbonate and water was then added to the starch solution and was continued to stir for an additional 1-2 min. The mixture was poured onto a baking pan in a thin sheet and placed in hot air oven to dry.

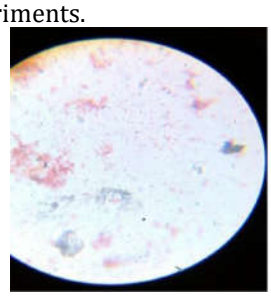
K. Characterization of PHA

The PHA produced was characterized with FTIR analysis, DSC and other mechanical properties.

RESULTS AND DISCUSSIONS

A. Sterilization techniques

Autoclave sterilized substrate showed better production as the substrate got partially degraded by autoclaving which was efficiently utilized by the micro-organism. Thus, autoclave sterilized substrate was chosen for further experiments.



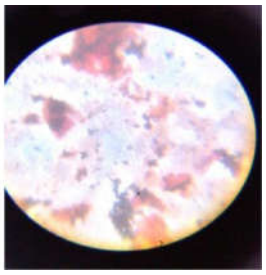


Fig I. Ethanol sterilized **Fig IIA**utoclave sterilized

B. Comparison between SSF and SmF Technique

Various flowers were tested for PHA production by the previous batch students of SIES GST. They found Marigold flowers as optimal for PHA production. So we have considered Marigold flowers as a carbon source for our study. PHA was produced by SSF and SmF techniques. SSF has better yield of PHA as shown in the figures below where more dense PHA granules were observed in SSF (65%) as compared to SmF (58%)

Table I. Cell dry weight analysis of SSF and SmF

Samples	PHA accumulation (%)
SSF	65.75
SmF	58.46

C. Comparison between various forms of substrate

Various forms of substrate like shredded Marigold flowers and powdered marigold were tested for PHA production and compared with glucose as a standard carbon source. Among all, the powdered substrate was found to give best result.

Table II. Cell dry weight analysis for various forms of substrate

Sample	PHA accumulation (%)
Glucose standard	34.62
Shredded substrate	48.15
Powdered substrate	65.88

D. Recovery of cells form fermented broth

Recovery of PHA was done from the fermentation broth by adding water after incubation and was subjected to vigorous stirring at various time intervals i.e half hour, one hour and one and half hour. One and half hour gave better yield of PHA (68%).

Table III. Cell dry weight analysis for recovery of cells form broth

Samples	Cell dry weight (g/250 ml)	Dry weight of PHA (g/250 ml)	PHA accumulation (%)
Half hour stirring	0.059	0.03	50.85
One hour stirring	0.067	0.039	58.21
One and half hour stirring	0.072	0.049	68.05

E. Production of PHA:

By optimization of all the parameters, optimized production of PHA is carried out. The optimized parameters are set as, Substrate – 8gm, Incubation time 24 hours, Initial moisture content – 83%, Inoculum level 7 ml, optimum temperture – 30^{o}C.

Table IV: Production of PHA at optimum conditions

Sample	CDW (mg/l)	Concentration (mg/l)	%PHA Accumulation
Actual	73	46	64.38
Duplicate	80	48	62.5
Average	76.5	47	63.44

F. YIELD OF PHA

The yield of PHA can be calculated by the following formula:

$$Yield = \frac{Quantity\ of\ cell\ dry\ matter\ product\ (mg)}{Quantity\ of\ carbon\ substrate\ utilized\ (gm)}$$

$$= \frac{47}{8}$$

Yield = 5.875 mg/gm of substrate

G. FTIR ANALYSIS

Polymer which was obtained after extraction was subjected to FTIR spectroscopy which showed intense absorptions typical to PHA at 1724–1745 cm^{-1} and at 1280 cm^{-1} corresponding to C=O and C-O stretching groups, respectively in all the four samples. The Spectrum obtained is shown in the figures below. These figures confirm the presence of PHA in the samples. [22]

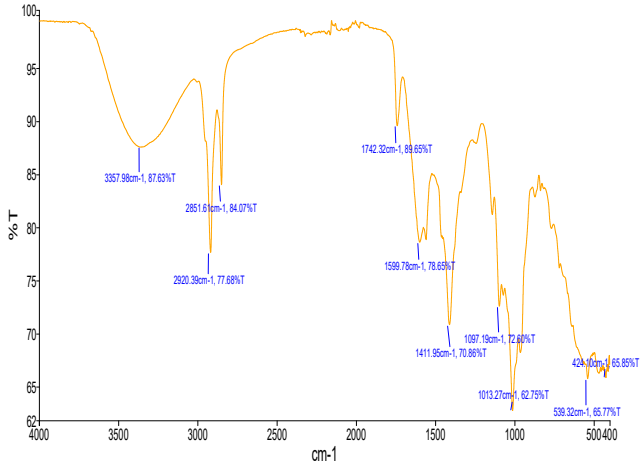


Fig III. FTIR analysis of produced PHA

Differential Scanning Calorimetry

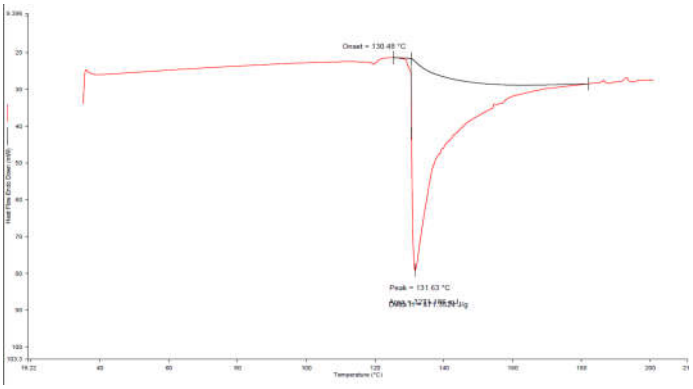


Fig IV. DSC of PHA

Table V: DSC thermogram for PHA and standard PHA

Sample	**Tm, ^{O}C**	**ΔH, J/g**
PHA	131.63	47.35
Standard PHA	176.20	86.49

CONCLUSION

Ralstonia eutropha was selected as the strains for PHA production from flowers since it can be grown in various substrates.A testing phase was performed where PHA was produced using both SmF and SSF techniques. Cocentration of PHA was found to be 38 mg/l in SmF and 48 mg/l in SSF. Since SSF showed better result it was chosen for further production of PHA.During the testing phase both fresh and waste Marigold flowers were used as a substrate for PHA production. PHA obtained from fresh Marigold was 48 mg/l and that from waste Marigold flowers was 45mg/l. Hence for cost efficiency waste Marigold flowers was chosen as a substrate for this study. Different forms of substrate (shredded Marigold and powdered form) were compared with standard values of glucose which are as followsGlucose – 9 mg/l, Shredded substrate – 26 mg/l, Powdered substrate – 56 mg/l. Cells were recovered from the fermentation broth by vigorously stirring the flask at various intervals from which one and half hour vigorous stirring gave better yield i.e 49 mg/l.The following parameters were optimized to maximize PHA productionCarbon source – 8 gm, Incubation Time – 24 hours, Initial moisture content – 83 %, Inoculum level- 7 ml, Temperature – 30^{o} C. With every step of optimization the yield of PHA obtained increased.The recovery of PHA from the cells was standardized for waste Marigold flowers as a substrate.The optimized PHA production was found to be 47 mg/l.The yield of PHA was found to be 5.875 mg/gm of substrate.The presence of PHA was confirmed in waste Marigold (powdered form) using FTIR spectroscopy. FTIR gave positive results with presence of PHB and PHV (components of PHA) in the graph for the strain.The production of PHA was also confirmed using UV-VIS spectroscopy by the crotonic acid test in all the samples. This test was used as a quantitative test.Hence, the Marigold in powdered formis a better producer of PHA with*R. eutropha* using Solid State Fermentation.

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