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Application Of Response Surface Methodology For The Production Of PHB From Cupriavidus Necator Using Calotropis Procera Extracts As Carbon Source

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Abstract: Calotropis procera is a weed plant that grows profusely throughout the tropical countries without any management. The oil was extracted from the dried stem and leaves of plant Calotropis procera using Soxhlet extraction column and used as carbon source for the growth of Cupravidus necator. This organism was fed in fed batch mode and the culture was utilized for the production of Polyhydroxybutarate (PHB). The microorganism was procured from MTCC Chandigarh [MTCC No-1472]. The fermentation time for the production of PHB was observed to be 72hr. The intracellular PHB from the cells was extracted using various organic solvents and analyzed using Fourier Transform Infra red spectroscopy [FTIR] and the peaks are compared with the standard peaks. The production was also carried out as per the runs designed by the Central Composite Design and 3D plots are deduced to describe the effect of pH and Temperature on PHB production.

I. INTRODUCTION

The increasing awareness of environmental hazards arising from plastic wastes, especially those originating from non-renewable natural resources, presents new opportunities for the development of bio-based and biodegradable polymer materials(Hiren V. Doshi *et al* 2012, M. Manikandan and R. Arumugam 2012, Hanisah Kamailah *et al* 2013). Among the readily biodegradable, non-toxic and environmentally friendly materials, polyhydroxyalkanoates (PHA) have received considerable attention in recent years (Srivastava Vivek Kumar 2011, Paramjit Singh 2011, Song Yuyang *et al* 2013). Polyhydroxyalkanoates (PHAs) are biodegradable polyesters which are synthesized by many bacteria. They are accumulated intracellular as carbon and energy reserves under certain conditions like, in the presence of excess carbon source(John H. Law, Aremu, M.O 2011). Commercial production of polyhydroxyalkanoates (PHA) is developing, but price of this polymer is high and its production efficiency is too low in comparison with petrochemical based plastics. These two factors are of concern in the pathway development of polyhydroxyalkanoates compared to the synthetic polymers such as polyethylene and polypropylene (Yu-Hong Wei *et al* 2011, Alyaa Hamieh 2013). *Calotropis procera* belongs to the family Asclepiadaceous, is a well known wasteland weed grows wild up to 2-5 m profusely throughout the country on a variety of soils and in different climates (Charles F. Budde, Shilalipi Samantaray 2011, S. Chaijamrus 2008). Several studies have been conducted in this plant species to evaluate its medicinal bioactive compounds and recently its latex content drawn the attention of researchers to evaluate its use in biomethanation. However, studies were limited on the identification of potential hydrocarbons and their role in Polyhydroxybuterate (PHB) production (Kemarajit Kemavongse 2007, Rob AJ Verlinden 2011). Enhancing the efficiency of PHB production process, involves precise expression of kinetic model for PHB production and its optimum parameters, including dry cell weight, product concentration and substrate consumption. The statistical model is capable of analyzing data and creating a strategy to resolve fermentation and product formation issues, and also being informative about fermentation process kinetic parameters, which should have the potential to increase production efficiency (Mohd. Zafar *et al* 2012). Response surface methodology (RSM) explores the relationships between several explanatory variables and one or more response variables. The main idea of RSM is to use a sequence of designed experiments to obtain an optimal response. By careful design of experiments, we can optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response (S.V.N. Vijayendra *et al* 2007). In this study, response surface methodology was applied for modeling of microbial production of PHB from *Cupriavidus necator* using *Calotropis procera* plant extracts as carbon source.

II. MATERIALS & METHODOLOGY

A. Extraction of oil

The leaves and stems of *Calotropis procera* are collected from Visakhapatnam city and sun dried for 11-15 days. These dried leaves and stems were made into fine powder using grinder and oil was extracted from this powder using Soxhlet extractor. The extracted oil was used as a carbon source.

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B. Culture conditions

The micro organism used for the production of PHB was Cupravidus necator and was procured from MTCC Chandigarh [MTCC No-1472]. The lyophilized culture is revived and was cultured in nutrient media. From the revived culture, stock culture was prepared and from the stock culture, starter culture was prepared. This starter culture was used for the production of PHB.

Optimization of parameters using Response Surface Methodology

C. Production

In the PHB production media, the oil extracted from the Calotropis plant was the carbon source (10%w/v) and other nutrients such as K2HPO4 (0.05w/v), MgSO4.7H20 (0.02%w/v), NaCl (0.1%w/v), FeCl3 (0.9%w/v) were added in the media used for the optimum production of PHB. To the prepared media, 5% starter culture was added and was incubated for 72 hrs with continuous shaking at 120rpm. After 72hrs the PHB accumulated inside the cells was extracted from the biomass.

D. Extraction of PHB

The bacterial cultures were harvested by centrifugation at 5000 rpm for 10 min. The cell pellet was suspended in sodium hypochlorite solution and incubated at 37°C for 2 hr for complete digestion of cell components except PHB, where lipids and proteins were degraded. The mixture was centrifuged to collect PHB granules and the supernatant was discarded. The sediment was washed twice with 10 ml of distilled water and centrifuged. The PHB granules in the sediment were washed twice with acetone, methanol and diethyl ether (1:1:1) respectively. The polymer granule was dissolved by boiling in chloroform and was evaporated by air drying, to yield dry powder of PHB (Preethi. R, Sasikala. P, Aravind 2012).

Quantification of PHB

The bacterial culture was centrifuged at 5000 rpm to obtain the cell pellet and dried to estimate the dry cell weight (DCW) in g/L. Residual biomass was estimated as the difference between dry cell weight and dry weight of extracted PHB. This was calculated to determine the cellular weight and accumulation of compounds other than PHB. The percentage of intracellular PHB accumulation is estimated as the percentage composition of PHB present in the dry cell weight.

Residual biomass (g/L) = DCW (g/L) – Dry weight of extracted PHB (g/L)

PHB accumulation (%) = Dry weight of extracted PHB (g/L) × 100%

DCW (g/L)

FTIR analysis

The extracted PHB samples were sent for FTIR analysis in Nanotechnology laboratories of GITAM University. The FTIR spectroscopic analysis gave further insights in to the chemical structure of the polymer and reflects the monomeric units.

III. RESULTS AND DISCUSSION

Optimization of parameters using Response Surface Methodology

The experiments were designed using central composite design by taking the parameters as input factors, the runs are performed experimentally. These runs give the effect of pH, temperature and fermentation time on the PHB production. The yield decreases with increase in temperature and pH.

Fig.1 The effect of pH and temperature on the yield

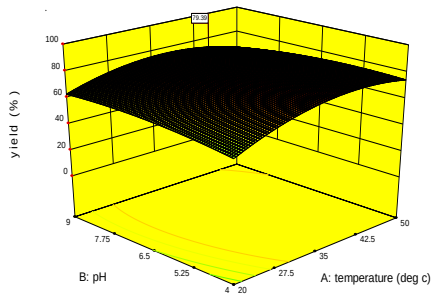

Fig.2 The effect of fermentation time and pH on yield

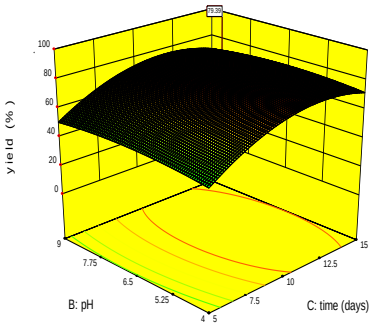

Fig.3. Effect of temperature and time on the yield

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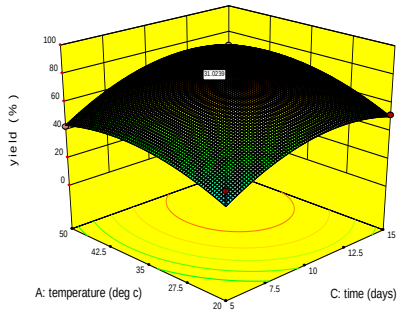


Final Equation:

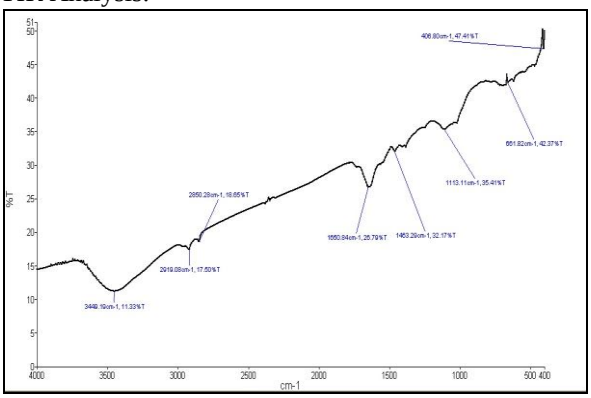
$$Yield = +85.51 + 0.78*A + 2.14*B + 1.32*C + 2.38*AB + 0.38*AC - 1.12*BC - 26.02*A^2 - 6.75*B^2 - 6.75*C^2$$

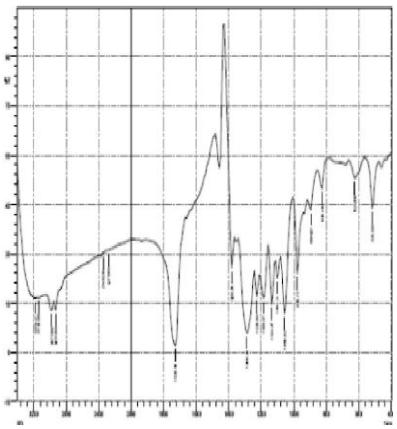
Where A, B and C are the independent variables. The significance of each coefficient present in Equation was determined by the F-values and values of probability >F. The F-value is the test for comparing the curvature variance with residual variance. If the variances are close to the same value, the ratio will be close to one and consequently it is less likely for the curvature to be significant. Probability >F (P-value) is the probability of seeing the observed F-value if the null hypothesis is true. Small probability values call for rejection of the null hypothesis that the curvature is not significant. The results of the quadratic model in the form of ANOVA showed small probability value ($P < 0.00001$) indicating the individual terms in the model have significant on the effect. The values of R-squared ($R^2 = 0.9241$) and adjusted R-squared (Adj. $R^2 = 0.8482$) are closed to 1, which is very high and indicates a high correlation between the observed and the predict. The predicted R-squared (Pred. R^2) of 0.8240 is in acceptable agreement with the adjusted R-squared of 0.8482. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 10.398 indicates an adequate signal. Therefore, this model can be used to navigate the design space. The examination of the fit summary output revealed that the quadratic model is statistically significant for the response and therefore it will be used for further analysis. The substrate which was used for the production of PHB was a novel substrate and was the cheapest, widely and abundantly available substrate. Based on the composition of the oil which is extracted from plant, which is rich in carbon content is the ideal source for the optimum production of PHB. As well as the fatty acid content of the oil also gives higher yield theoretically. The time required for the production of PHB can be varied from 72hrs to 96hrs. The optimum time for the production was usually at 72hrs, but sometimes it might extend till 96hrs. After the peak time the media will start depleting the carbon source, so the bacteria will consume the produced PHB as carbon source. So for the optimum accumulation of PHB the extraction should be done at 72hr.

Quantification of PHA

Extracted PHA was quantified and PHA accumulation was observed as 41.6% Dry weight of extracted PHB=.054 g/ml Cell dry weight =.111g/ml Residual biomass weight =.057g/ml

FTIR Analysis:





In this study the functional groups of the polymer PHB was confirmed as C=O groups by FT-IR spectroscopy. The FTIR spectrum of the extracted PHB sample was compared with that of the Standard Polyhydroxy-3-Butyric acid. From the spectra, sample bands were observed similar with the standard spectra and according to the results obtained by Oliveira *et al*., (Oliveira *et al* 2007) in the regions of 1650.48, correspond to the stretching of the C=O bond whereas a series of intense bands located at 1113.11 cm -1, which was found exactly similar with the standard spectra and correspond to the stretching of the C–O bond of the ester group. The sample bands were observed similar with the standard spectra and according to the results obtained by Pandiyan *et al*., (Pandiyan *et al* 2010) in the region of 661.82 cm -1 correspond to the presence of the C=O bond, 3449.19 cm^{-1} corresponding to the hydroxyl (OH) stretching, 2919.08

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cm^{-1} (CH,CH_2 CH_3) ,1463.29 corresponding to ester carbonyl groups present in hydroxyl acids. Two peaks in the region of 1113.21 and 1650.48 were exactly the same in the Standard PHB, confirming the presence of PHB in the extracted PHB sample. The FTIR spectra of the extracted PHB and the standard PHB sample are given in figures 4 and 5.

IV. CONCLUSION

Cupriavidus necator is the efficient culture which can consume plant oil and efficiently produce PHB. The plant oil which is extracted from calotropis procera is widely, abundantly and cheaply available carbon source which is effectively used by cupravidus necator for the efficient production of phb. The production is maximum at optimum process conditions of 72-76 hours of production time, at a temperature between 25^0C and at a pH 6. The FTIR analysis confirms the PHB production.

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