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Research Article

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Production and characterisation of biopolymer using *Pseudomonas fluorescens (pf 01)*

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ABSTRACT

There is worldwide concern regarding the development of biodegradable polymer as a remedy towards harmful effects caused by synthetic polymer. These materials which are synthesised by petrochemical process, contribute towards air pollution and waste management problem. Biodegradable polymers are able to significantly reduce the environment impact in terms of energy consumption and greenhouse effect. Biopolymers are synthesised by microbial sources and this study aims to produce biopolymer from Pseudomonas fluorescens (pf01) by fermentation process. Biopolymers produced by bacterial species were screened by Sudan Black B staining method. The polymer extraction was carried out by Sodium Hypochlorite method. The maximum biopolymer production of 55% was observed at 72 hrs. FTIR and DSC methods were carried out for the characterisation process.

Keywords: Biopolymer, *Pseudomonas fluorescens*

INTRODUCTION

Polymers are the larger macromolecules with repeating units of monomer. They are solid, higher molecular weight, non-metallic compounds. Each polymers have different characteristics depending upon their number and arrangement of monomer units [1]. The major unique properties of polymers includes toughness, viscosity, elasticity and semicrystallnity. The polymers are classified into synthetic polymers and biopolymers respectively. The biopolymers are overlooked by synthetic polymers in terms of its biocompatibity and biodegradability. The advantages of synthetic polymers over biopolymers include low production cost, stability, durability, mechanical stability, thermally stability and they can be tailored easily into any useful product [2]. Biopolymers are biological macromolecules which can be derived from renewable resources such as plants, animals and living organisms. The examples are silk, wool, xanthan, pullulan etc. [3].

Biopolymers are easily facilitated by natural degradation mechanism. The time required for degradation of synthetic polymers may take years which is very much higher when compared to biopolymer degradation time i.e.47 to 90 days. Hence, these biopolymers offer an opportunity to create a sustainable environment and an efficient carbon dioxide mitigation strategy [1]

The biopolymers are classified into three main categories such as amino acid based biopolymers, polysaccharide from bacteria and polyester. Amino acid based biopolymers include plant proteins (Corn proteins, soy proteins, wheat proteins), animal proteins (Casein, collagen, gelatin, keratin) [4]. Natural polysaccharides are biologically produced polymeric materials which possess hydrophobic, glycosidic linkages. The examples of polysaccharides include pectin, xanthan gum, succinoglycan, alginate [5]. PLA and PHA are bio based polyesters where PLA are very hard, hydrophilic, resistant against oil based products and act as flavour/odour barrier for food stuffs [3].PHAs

are intracellular bacterial polymers which can be employed in biomedical applications such as drug encapsulation, controlled drug delivery mechanism, porous structure for tissue engineering etc.

Many microorganisms have been reported on the production of biopolymers such as *Ralstonia eutropha, Bacillus megaterium, Pseudomonas sp., Recombinant E.coli, Enterobacter aerogenes*. In the present study, biopolymer producing ability of *Pseudomonas fluorescens* was reported for the first time and biopolymer was produced and characterised using Fourier transform Infra-red spectroscopy(FTIR) and Differential scanning calorimetry(DSC) respectively.

EXPERIMENTAL SECTION

Bacterial strain

The *Pseudomonas fluorescens (pf 01)* strain used for the biopolymer production was obtained from Tamil Nadu Agricultural University, Coimbatore. The powder form of *Pseudomonas fluorescens (pf 01)* sub cultured by using Kings' B (KB) medium. The sub cultured bacteria inoculated in Yeast Peptone (YP) medium for 24 hours at 150rpm in the shaker.

Gram Staining

The gram stain is a differential technique which is used to classify and categorize bacteria into two major groups (i) gram positive bacteria (ii) gram negative bacteria. The isolated culture was characterized based on Gram's staining method

Sudan Black B Staining

Isolated culture was characterized as biopolymer positive (+ve) or biopolymer negative (-ve) based on Sudan Black staining of the cells taken from seed culture. Here the biopolymer positive (+ve) cells picked up the Sudan black stain and appeared dark in colour when observed under the light microscope, whereas negative cells were stained by the counter strain (safranin) only and they appeared pink. [6]

Fermentation Process

The 10 ml of seed culture was inoculated into the fermentation medium (Glucose – 20 g/l, Di ammonium sulphate($(NH_4)_2SO_4$) - 2.0 g/l, Potassium dihydrogen phosphate (KH_2PO_4) - 13.3 g/l, Magnesium sulphate ($MgSO_4.7H_20$) - 1.2 g/l, Citric acid - 1.7, Manganese sulphate ($MnSO_4$)- 0.4, Ferrous sulphate ($FeSO_4.7H_20$) - 1.0g/l, $CoCl_2.6H_2O$ - 0.2 g/l, Zinc chloride ($ZnCl_2$) - 0.2g/l) which was incubated at 37°C, 72hrs.[7]

DNSA method:

The method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in glucose. Simultaneously, 3, 5-dinitrosalicylic acid (DNS) is reduced to 3-amino, 5-nitro salicylic acid under alkaline conditions. Amount of carbon utilized by bacteria is analysed by this method. Carbon concentration reduced during the course of fermentation is measured.

Extraction of Biopolymer

Intracellular biopolymer was extracted by using Sodium hypochlorite extraction method. Initially 50ml of incubated fermentation medium was taken and centrifuged at 6000 rpm for 15 mins and the supernatant was discarded. The cell pellets were suspended in lM of sodium hypochlorite and incubate for 1-2hrs at 37°C for complete digestion of cell component except biopolymer, where by lipids and proteins were degraded. This mixture was centrifuged at 6000 rpm for 15 mins and the supernatant was discarded. The sediment was washed twice with distilled water and centrifuged. The biopolymer granule was washed with acetone. The biopolymer was dissolved with boiling chloroform and was evaporated by air drying to yield dry powder of biopolymer. [7, 8]

Characterization of Biopolymer

The polymer content of the cell and composition of biopolymer were determined by using the following methods

Fourier Transform Infra-Red Spectroscopy (FTIR)

FTIR was carried out using Perklin Elmer Spectroscopy. The extracted biopolymer sample was added with KBr. This sample was subjected to FTIR, to analyse the biopolymer structure. The Peaks were observed from 4000-400cm^{-1}.

Differential Scanning Calorimeter (DSC)

The thermal treatment of biopolymer powder was carried out by using DSC Q20 V24.10 Build 122(Universal V4.54 TA instrument). The Differential Scanning Calorimeter had one sample cell and one reference cell samples (<10mg) were exposed to a temperature 0-500°C.

RESULTS AND DISCUSSION

Revival of Organism

The lyophilized form of Pseudomonas *fluorescens (pf 01)* was sub cultured using Kings'B (KB) medium [9].

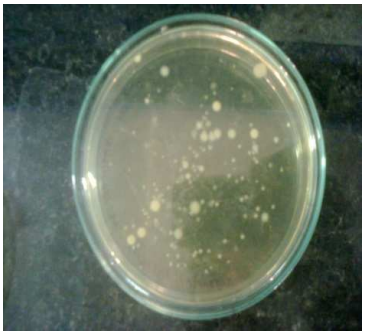

Fig 1. *Pseudomonas fluorescens (PF01)*

Fig 2. Subculture of *P.fluorescens (pf01)*

Gram Staining:

Gram staining method was used to confirm whether it is gram negative or gram positive bacteria. By using Gram staining method, PINK colour rod shaped bacteria was obtained and it was confirmed that the *Pseudomonas fluorescens (pf01*) is a Gram Negative Bacteria.

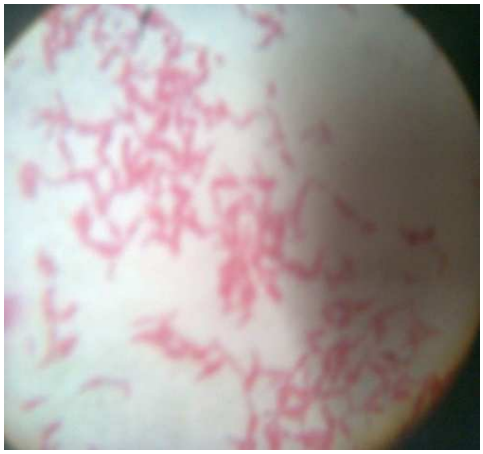

Fig.3.Gram staining method

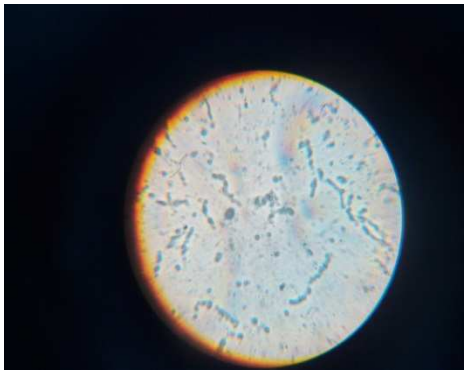

Fig.4.Sudan black B Stainning(Black colonies)

Sudan Black B staining method

Sudan Black B Staining was used to confirm whether the *pseudomonas fluorescens (pf01)* is capable of producing intracellular granules of biopolymer. Black coloured colonies were observed and it was confirmed that bacteria possesses biopolymer as granules [6].

Growth characteristics of *Pseudomonas fluorescens* (*pf01)*

Growth characteristics of Pseudomonas *fluorescens* was studied by measuring the absorbance at 600 nm for every 3 hrs time interval over a period of 90 hrs using UV-Visible spectrophotometer and curve was plotted between A_{600nm} versus time.

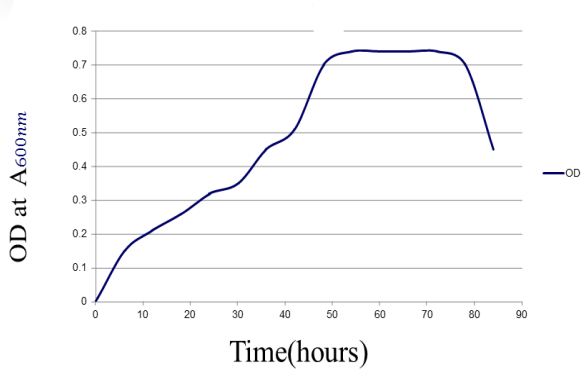

Fig.5.Growth curve of *Pseudomonas fluorescens (Pf 01)*

Recovery of product (biopolymer)-sodium hypochlorite method

Sodium Hypochlorite method was used to extract the biopolymer intracellularly. We obtained WHITE colour crystal shaped product and biopolymer was characterised using FTIR and DSC. [9]

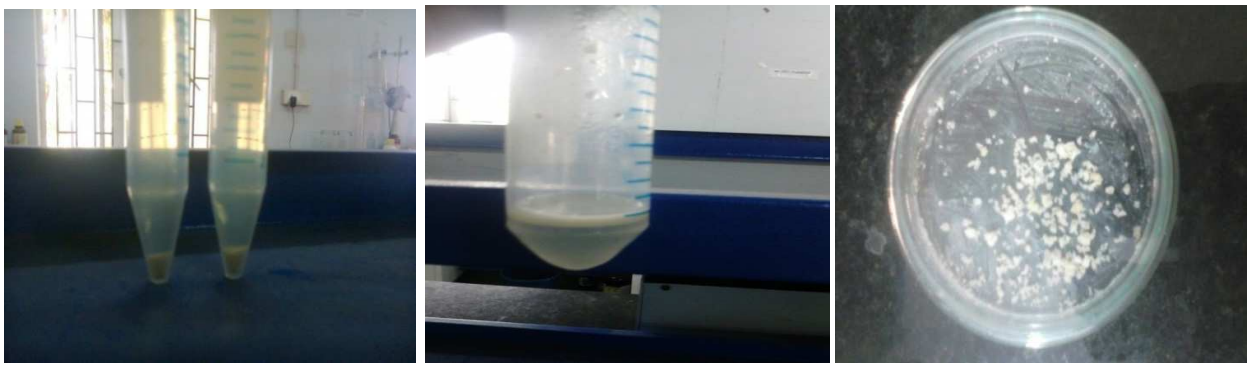

Fig 6.Biopolymer

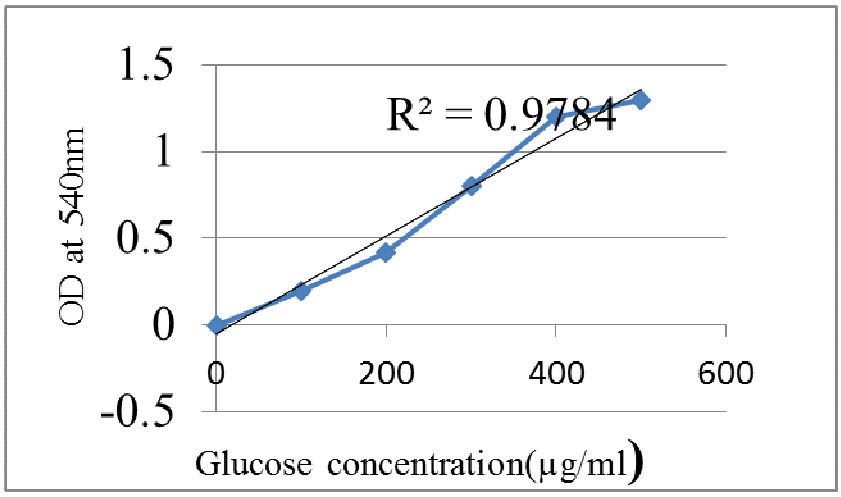

Fig 7- Standard graph for glucose concentration

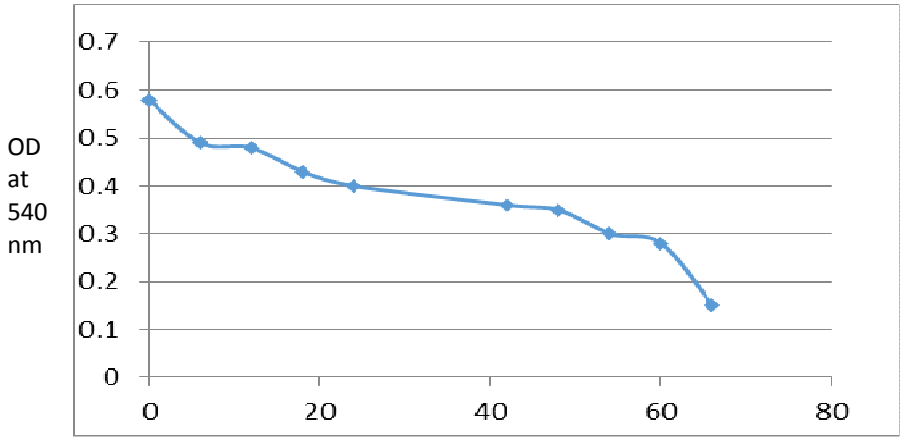

Fig 8-Reducing sugar concentration for product

DNSA Method

DNSA method was performed to study the utilization of substrate during the course of fermentation**.** Reducing sugar concentration was measured at every 6 hours. At 66th hr, there was a decline in reducing sugar concentration value meanwhile the cell growth was increased.

YIELD DETERMINATION OF BIOPOLYMER

10 ml of seed medium was transferred aseptically to the production medium and incubated for 72 hrs in incubator shaker at 250 rpm. Meanwhile, the cells were subjected to chloroform-Sodium hypochlorite extraction method as shown in fig. 6 and yield was calculated using the formula given below.

$$\text{Yield \%} = \frac{\text{Amount of Biopolymer}}{\text{Amount of biomass}} \times 100$$

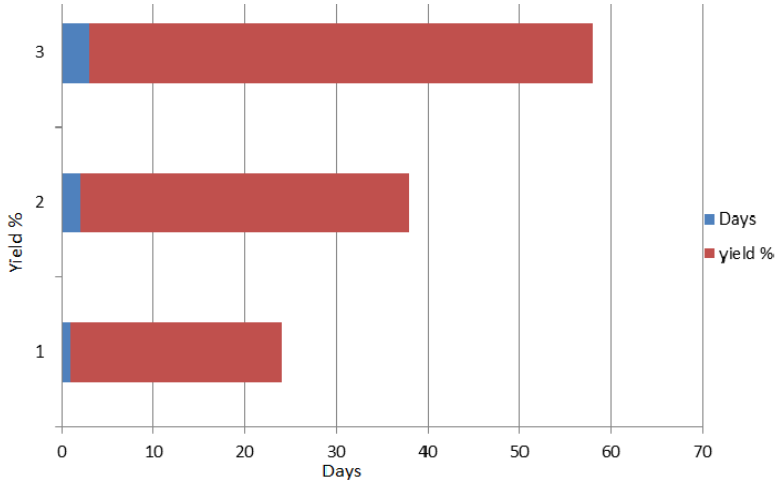

Fig 9. Yield of Biopolymer during the Course of Fermentation

CHARACTERIZATION OF BIOPOLYMER

FT-IR Analysis

FTIR spectra of biopolymer obtained from *pseudomonas fluorescens (pf01*) were recorded with a Perkin Elmer Spectrum Version 10.4.2 of Fourier transform infrared spectrometer. The spectral region between 4000 and 400 cm-1was scanned. Specimen was prepared by mixing the KBr and biopolymer sample, pressed in vacuum to homogeneous disc with a thickness of 0.5 mm. FTIR spectrum was obtained and it was compared with FTIR spectrum of chitosan obtained from housefly larvae reported earlier. [10]

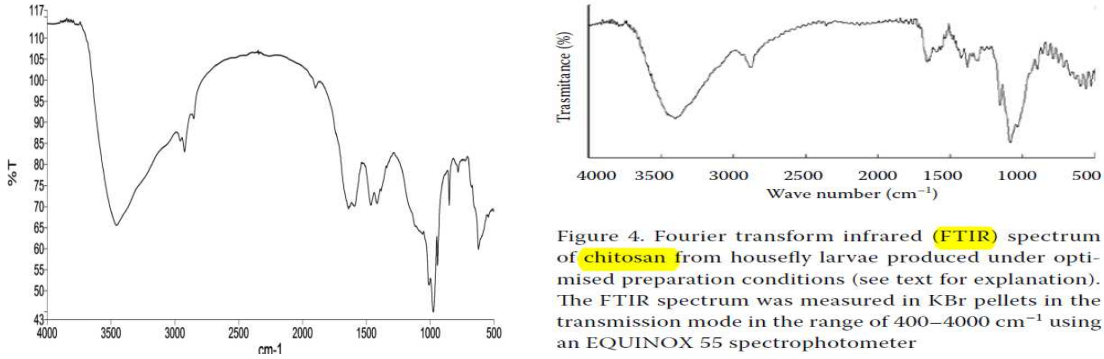

Fig. 10. FTIR Analysis a) Extracted biopolymer b) Chitosan from housefly larvae [10]

Table 1-Funtional group of product

WAVENUMBER (cm^{-1})	FUNCTIONAL GROUPS
1021.37	C-O-C
1409	Joint Contribution Of OH,CH
1604	C=N
1628	Amide
1715	CHO
3400	AMINO PEAK
3454	OH

The IR spectra of chitosan showed a strong absorption band at 3454 cm^{-1} , 1715 cm^{-1},1604 cm^{-1}, corresponding to symmetrical stretching vibrations of OH , -CHO, C=N respectively. Yeng *et al* (2013)[11] reported that broad peak at 3800cm-1 to 3000cm^{-1} was assigned to –OH group, peak at 2920cm-1 was observed due to –CH stretching. Meanwhile, peaks of 1634cm^{-1} and 1550cm^{-1} indicated the –NH bending of NH_2. Similar observations were also reported by Moreno – Osorio *et al* (2010) [12].

DSC Analysis

From the DSC, the appearance of endothermic event between 108 and 245 ˚C was observed. This peak is attributed to the loss of water associated with the hydrophilic groups of the polymer. The exothermic peak, which appears in the temperature range between 250 and 370 ˚C, corresponds to the decomposition of the polymer [13, 14] using DSC Q20 V24.10 Build 122. Tarane Gazari et al (2009)[15] reported that the DSC thermogram of Chitosan showed an endothermic peak at 82.49˚C and a higher exothermic peak at 308.03˚C. Endothermic peaks were correlated with loss of water attributed to hydrophilic groups of polymers while exothermic peaks result from degradation of polyelectrolytes due to dehydration and depolymerisation reactions. In specific, partial decarboxylation of the protonated carboxylic groups and oxidation reactions of the polyelectrolytes were occurred during this degradation and depolymerisation mechanism.

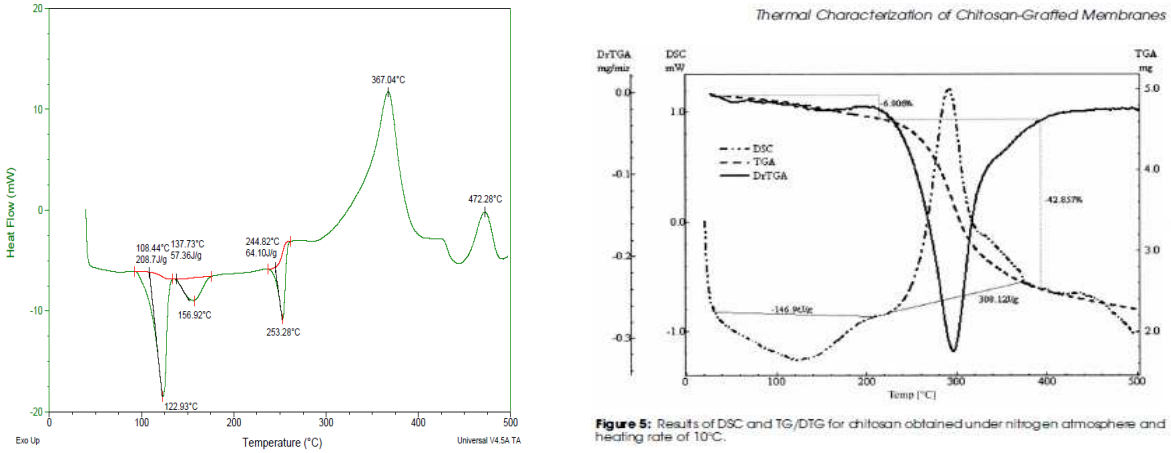

Fig 11. DSC Analysis (a) Extracted Biopolymer (b) Chitosan from housefly larvae [10]

CONCLUSION

This study has explained about the production and characterization of biopolymer. The product was isolated by using Sodium Hypochlorite method and FT-IR, DSC methods were employed for the characterization of the product. The yield % of chitosan obtained was 55%. The characterization studies revealed that the product resembles to the chitosan biopolymer suggesting that the extracted biopolymer to be "CHITOSAN".

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