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Research Article

Extraction, Characterization and Preparation of Microbial Polymer and Polymer Layered Composite from Textile Dye Degraded Bacterial Biomass

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ABSTRACT

The present study includes the use of potent dye degrader bacteria *Providencia spp. RMG1* and *Bacillus spp.RMG2* for the production of biopolymer poly 3 - hydroxyl butyrate (PHB). Individually and in consortium of these strains were used for extraction of PHB. Different agricultural waste like whey waste, molasses, bagasse, rice bran were used and studied for the production of PHB in the presence of dye. Molasses and whey waste were shown the higher production of PHB at optimum pH7 and optimum temperature 37^{o}c and 30^{o}c, respectively under static anoxic condition. Acrylic acid was used as a inhibitor and optimized in different concentration for intermediate of β-oxidation pathway of fatty acid biosynthesis for increasing the PHB production.PHB was extracted from bacterial cells and estimated by crotonic acid assay. PHB synthase activity was also observed. Bio-composite of PHB and montmornolite organo clay was prepared. Antimicrobial activity of bio-composite was studied against *Escherichia coli* and *Staphylococcus aureus* microbial culture. Thermo stability was found more for microbial PHB than standard PHB. Extracted PHB sample was characterized by HPLC, FTIR and GCMS analysis.

Keywords: - Reactive red 4E8Y5, poly 3 - hydroxyl butyrate, bio-composite, *Providencia spp. RMG1*, *Bacillus spp.RMG2*, acrylic acid.

I INTRODUCTION

PHAs comprise a class of polyesters accumulated by microorganisms as a stored reserve of carbon, energy, and reducing power [1-3]. PHB is a partially crystalline material with a high melting temperature. Most of the current research is on biodegradation of textile dyes but there is a very less research on use of biomass left over after biodegradation. This biomass can be used for production of bacterial polymer like PHA. Left over biomass is a good sourse of bacterial polymer and reduce the cost of the process which make biodegradation economic for textile dye industry [4]. Bioplastic like Polyhydroxy butyrate produce by bacteria in stressed conditions like carbon or nitrogen limiting condition. It was also reported that consortium of three organisms (*Sphingobacterium sp. ATM, Bacillus odysseyiSUK3, and Pseudomonas desmolyticum NCIM 2112*) were used for degradation of textile dye Orange 3R and production of PHAs along with individual strains [5]. PHB production can also be enhanced by using extracellular electron transfer method [6].

During the last decade, interest in polymer layered silicate (PLS) has rapidly been increasing at an unprecedented level, both in industry and in academia, due to their potential for enhanced

physical, chemical, and mechanical properties compared to conventionally filled composites[7]. Biocomposite have the potential of being a low-cost alternative to high-performance composites for commercial applications in both the automotive and packaging industries. Polymer composites are two-phase materials in which the polymers are reinforced by nanoscale fillers. The most heavily used filler material is based on the smectite class of aluminum silicate clays, of which the most common representative is montmorillonite (MMT) [8]. Most of the Layered silicates engineering polymers are hydrophobic [9].Antimicrobial silver silica nanocomposite also shown antimicrobial activity [10].

It is well established that when layered silicates are uniformly dispersed (exfoliated) in a polymer matrix, the composite properties can be improved to a dramatic extent. These improvements may include increased strength, higher modulus, thermal stability, barrier properties, and decreased flammability [11].

II METHODS

2.1 Isolation, Screening and identification of microorganisms

Microorganisms were isolated from soil contaminated with textile processing and dye manufacturing unit in Hatkanangle (India), by an enrichment culture method. The morphologically distinct bacterial strains were selected for the dye decolourization study. Two potent dye decolorizing bacterial strains were identified on the basis of their morphologically, biochemically and 16s ribosomal RNA gene sequence analysis.

2.2 Bacterial culture conditions

Pure cultures of isolated microorganisms were maintained at 4^{o}c and sub culture monthly on nutrient agar slants. The pure culture of *Providencia spp. RMG1* (PS) and *Bacillus spp. RMG2(BS)* and consortium of these two (PB1) were grown in 250 ml Erlenmeyer flask, containing 100 ml nutrient broth containing (gl^{-l}) peptone 10, Nacl 5, beef extract 3. Pure cultures of isolated microorganisms were also show the optimum pH7 at 37^{o}c and 30^{o}c for 24 h, respectively under static anoxic condition.

2.3 Development of Bacterial consortium PB1

The consortiumPB1 was developed by aseptically transfer of 2.5 ml suspension of overnight grown culture of each bacterial strain i.e. *Providencia spp. RMG1* and *Bacillus spp. RMG2* in 100 ml nutrient broth, optimum pH7 at 37^{o}c for overnight, under static anoxic condition. Individual strains used in this study *Providencia spp. RMG1* and *Bacillus spp. RMG2* were inoculated with 1 ml suspension of 24h grown culture respectively, to maintain the same cell count in the pure culture and it's consortium.

2.4 Media preparation by using different agricultural waste

The individual strains and the developed consortium PB1 were first grown on nutrient broth (100ml) to determine PHB production and later optimized with different carbon and nitrogen sources using wastes like baggase powder, whey waste, molasses and rice bran with different concentrations. Baggase powder and rice bran were mixed with 100ml distilled water individually, boiled for 15mins, cooled and filtered after boiling. The boiled extract of each agricultural waste was used as an inducer in different proportions with beaf extract broth for enhanced production of ligninolytic enzymes. The mixture of boiled extract and media was sterilized by autoclaving at 121˚C for 15min and then used for the growth of the individual strains and the developed consortium PB1. Media with different concentrations of whey waste and molasses were prepared individually and sterilized similarly as earlier and used for growth of microorganisms.

The optimum temperatures and pH for the growth of the strains were maintained. The dye reactive red 4E8Y5 ($50mg^{-l}$) was added to each culture flask at the exponential phase and incubated under static condition.

2.5 Dyestuff and chemicals

Reactive red 4E8Y5, disperse blue 34,disperse yellow 5RX, disperse brown3REL, reactive orange HEZR, reactive golden yellow HER, reactive orange TGLL, reactive green HE4BD, reactive yellow GR were generous gift from Yashwant textile industry Ichalkaranji, India. All chemicals were of the highest purity and of an analytical grade. 2, 2 108 0-Azinobis (3-

ethylbezthiazoline-6-sulphonate) (ABTS) was purchased from Sigma Aldrich, USA. Sodium hypochlorite, chloroform and crotonic acid were purchased from Himedia Research Laboratories, India.

2.6 Decolourization experiments

The 24 hrs grown consortium-PB1 and individual strains cells were incubated with different textile dyes such as Reactive red 4E8Y5, disperse blue 34,disperse yellow 5RX, disperse brown3REL, reactive orange HEZR, reactive golden yellow HER, reactive orange TGLL, reactive green HE4BD, reactive yellow GR at concentration, 50 mg l^{-1}, individually and incubated at 37^{o}C under static condition. Decolourization performance of mixture of above industrial dyes with concentration (50 mg l^{-1}) each was studied in 250 ml Erlenmeyer flask containing100 ml nutrient broth at 37^{o}C under static condition. An aliquot (3 ml) of the culture media was withdrawn at the different time intervals. Aliquot was centrifuged at 8000 rpm for 15 min to separate cell mass. Supernatant was used to determine decolourization by measuring the change in absorbance of culture supernatant at the maximum absorption wavelength (λ max) of the respective dyes.

The individual strains *Providencia spp. RMG1* and *Bacillus spp. RMG2* (PS and BS) and the developed consortium-PB1 cells grown in the nutrient broth for 24 h were used to monitor decolorization of Reactive red 4E8Y5 at 37^{o}C under static anoxic and shaking (120 rpm) condition. Decolourization at different initial concentrations of Reactive red 4E8Y5 (50–200 mg l^{-1}) were tested by using individual strains as well as developed consortium-PB1 at 37^{o}C under static condition. Studies on the effect of temperature (30–65^{o}C) and pH (3-9) were carried out in the nutrient broth (dye concentration, 50 mg l^{-1}) under static anoxic condition. Further, decolourization of repeated addition of dye aliquots (50 mg l^{-1}) to culture media was also studied in nutrient broth under static condition without supplement of additional nutrients. Studies on the effect of various carbon and nitrogen sources were carried out in the synthetic medium (dye concentration, 50 mg l^{-1}) at 37^{o}C under static condition by using developed consortium-PB1.1 gram bagasse powder, rice bran and wood shavings were mixed with 100 mL distilled water individually , boiled for 15 mins, cooled and filtered after boiling. The mixture of boiled extract and media was sterilized by autoclaving at 121^{0}C for 15 mins and then used for the growth of the individual strains (*Providencia spp.RMG1* and *Bacillus spp.RMG2*) and the developed consortium PB1.For this 10% inoculum with an optical density of 1.0 (at 620 nm), grown in the nutrient broth for 24 h was used for inoculation of synthetic medium. All decolourization experiments were performed in triplicates. Abiotic controls (without microorganism) were always included. The percentage decolourization was calculated [12] as follows:

$$\% \text{ Decolourization} = \frac{\text{Initial absorbance - Observed absorbance}}{\text{Initial absorbance}} \times 100\%$$

The average decolourization rate (μg h^{-1}) was calculated [13] as follows:

$$\text{Average decolourization rate} = \frac{C \times \%D \times 1000}{100 \times t}$$

Where C is the initial concentration of dye (mg l^{-1}) and %D is the dye decolourization (%) after time t (h).

2.7 Extration of Polyhydroxybutyrate from dye degrading biomass

Dye degrading bacterial suspension was centrifuged at 6000rpm for 10min. Then the pellet was washed once with 4ml of saline and again centrifuged at 6000rpm for 10min to get the pellet. Cell pellet was then suspended in 2ml of sodium hypochlorite (4% active chlorine) and incubated at 37˚C for 10min with continuous stirring. The extract was centrifuged again at 8000rpm for 20min. The pellet was then washed with 5ml of boiling chloroform solution. Chloroform extract was dried to get white powder of PHA which was estimated [14].

2.8Enzyme analysis

2.8.1 PHA synthase activity

Free thiols released during polymerization were determined by DTNB assay. The formation of insoluble granules was monitored semi quantitatively by continuously measuring the turbidity at 600nm in a quartz cuvette. The

turbity of the solution increases as granules are formed; resulting in the correspondence increase of the Optical Density at 600nm.A 5µl aliquot of the reaction mixture was quenched in 20µl of 5% trichloroacetic acid. DTNB buffer (1ml, 100mM Tris pH 8, 1mM DTNB, A_{412}=13.6 absorbance units $cm^{-1}mM^{-1}$) was added and thiols were determined spectrophotometrically at 600nm [15].

2.8.2 Enzyme activity during decolourizations

Decolorization of dyes occurred in bacterial cells containing media cells are because of the biotransformation enzymes viz. laccase, azo-reductase and tyrosinase. These enzyme activities were observed in the individual strains (*Providencia spp. RMG1* and *Bacillus spp.RMG2)* and the developed Consortium PB1.

2.9 Preparation of an organoclay:

20g montmorillonite was dispersed into1000 ml of hot water (80ºC) with continuous stirring. 7.7g octadecylamine and 2.9g of hydrochloric acid were dissolved into 500 ml of hot water (80ºC) and poured into the hot clay water dispersion under vigorous stirring for 50 min to flocculate the clay. The precipitate was collected by centrifugation at 10000 rpm for 15 min. It was then washed three times with 500 ml of hot water (80ºC), each time centrifuged at 10000 rpm for 15 min. On drying, the Organoclay was formed [16].

2.10 Preparation of polymer layer nanosilictae of montmorillonite and PHB:

Fine powder was prepared from 5% of starch, 0.5 % of PHB (poly hydroxyl butyrate), and 0.5 % modified montmorillonite by crushing properly. To the above mixture 1ml of acetic acid, 1ml of glycerin and 15ml distilled water was added .This entire mixture was heated up to 120°C on a burner until a thick paste was obtained. After this entire mixture was pour on a petri plate and allow drying for about 24hrs and biopolymer were obtained. Likewise different biopolymers were prepared of 0.7%, 0.15%, and 0.25% PHB by changing concentration of starch, modified clay, distilled water, vinegar and glycerin.

2.11 Antimicrobial activity of the polymer layered nanosilicate:

Nutrient agar plates were prepared with 2.5% agar. The 24hrs grown pure suspension of *Escherichia coli* and *staphylococcus aureus* was spread onto the nutrient agar plates using spread plate technique. The disc of polymer layered nanosilicate sheet was placed on the petriplate. It was then incubated at 37ºc for 24hr. After 24 hours incubation, Zone of inhibition was observed and measured on to the nutrient agar plates containing microbial cultures.

III. RESULT AND DISCUSSION

3.1 PHA Production during decolourization of dye Reactive red4E8Y5

Table 1 %PHB (with Reactive red 4E8Y5 dye degraded biomass) (w/w):

Microorganism	*Providencia sp.RMG1*	*Bacillus sp.RMG2*	Consortium
Nutrient Broth	8.46 ± 0.05	9.33 ± 0.08	9.23 ± 0.04
Whey Waste (2gm)	5.84 ± 0.012	15.91 ± 0.03	11.20 ± 0.01
Mollases(3 gm)	12.04 ± 0.03	18.42 ± 0.08	9.56 ± 0.04
Baggase (2gm)	0.187 ± 0.009	0.227 ± 0.010	0.144 ± 0.003
Rice Bran(2gm)	0.054 ± 0.007	0.82 ± 0.04	0.129 ± 0.02

Values are means of three sets ± SEM

Table 2 PHA synthase activity with different Lignocellulose waste

Lignocellulose waste	Strains	Intracellular (cell pellet)
Nutrient Broth	Consortium PB1	0.1583 ± 0.0077
	Providencia spp.RMG1	0.1263 ± 0.0204
	Bacillus spp.RMG2	0.183 ± 0.0115
Bagasse Powder	Consortium PB1	0.1597 ± 0.0132
	Providencia spp.RMG1	0.1973 ± 0.0155
	Bacillus spp.RMG2	0.2303 ± 0.0091
Rice bran	Consortium PB1	0.105 ± 0.0136
	Providencia spp.RMG1	0.0727 ± 0.006
	Bacillus spp.RMG2	0.8147 ± 0.02639

Molasses	Consortium PB1	0.1797 ± 0.0189
	Providencia spp.RMG1	0.2917 ± 0.0059
	Bacillus spp.RMG2	0.2467 ± 0.0126
Whey waste	Consortium PB1	0.0743 ± 0.0123
	Providencia spps	0.2307 ± 0.0253
	Bacillus spps	0.1583 ± 0.0077

($Uh^{-1}mgprotein^{-1}$) **Values are means of three sets** ± SEM

Table 3 PHAs production at 48 h growth during decolourization of mixture of dyes (Effluent from five Star MIDC Kolhapur)

	Providencia sp.RMG1	*Bacillus sp.RMG2*	Consortium
ADMI removal ratio (%)	60.3 ± 0.1471	84.81± 1.071	85.64 ± 0.3278
PHA Production at 48 h (gl^{-1})	0.55 ± 0.0787	1.2 ± 0.134	1.45 ± 0.0984
% dry weight	30.363 ± 0.2683	45.05 ± 0.438	48.38 ± 0.2735

Values are means of three sets ± SEM

Table 4 Effect of acrylic acid inhibition on PHA production Acrylic acid concentration µl/50ml

Acrylic acid concentration	PHA production % dry weight of microbial biomass
12.5 µl	48.37
10 µl	9.55
7.5 µl	**57.73**
7.2 µl	22.69
7 µl	29.86
6.5 µl	18.5

3.3 Poly hydroxy alkynoate (PHA) Characterization:

Fig. 1 FTIR Graph of PHA synthesized on Activated sludge as a medium-

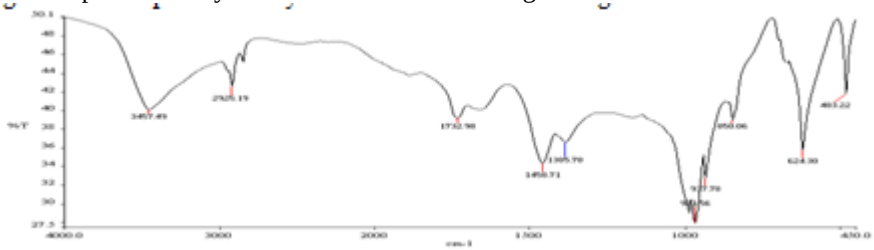


Fig. 2 FTIR Graph for PHA synthesized on Molasses as a medium-

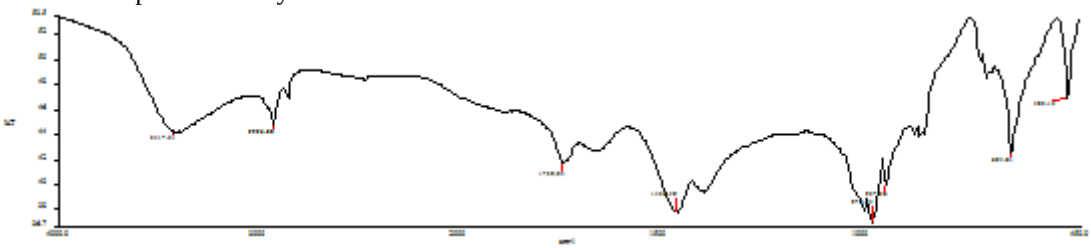


Fig.3 HPLC Profile of 1) standard and 2) Extracted Poly Hydroxyl Alkynoate (PHA)

High Performance Liquid Chromatography (HPLC) analysis:

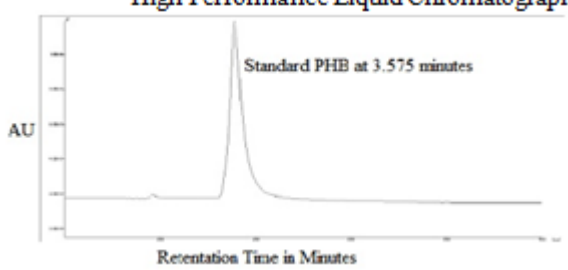


Fig 3.1

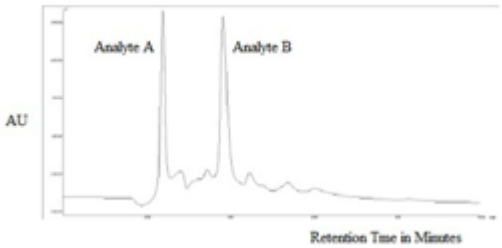


Fig 3.2

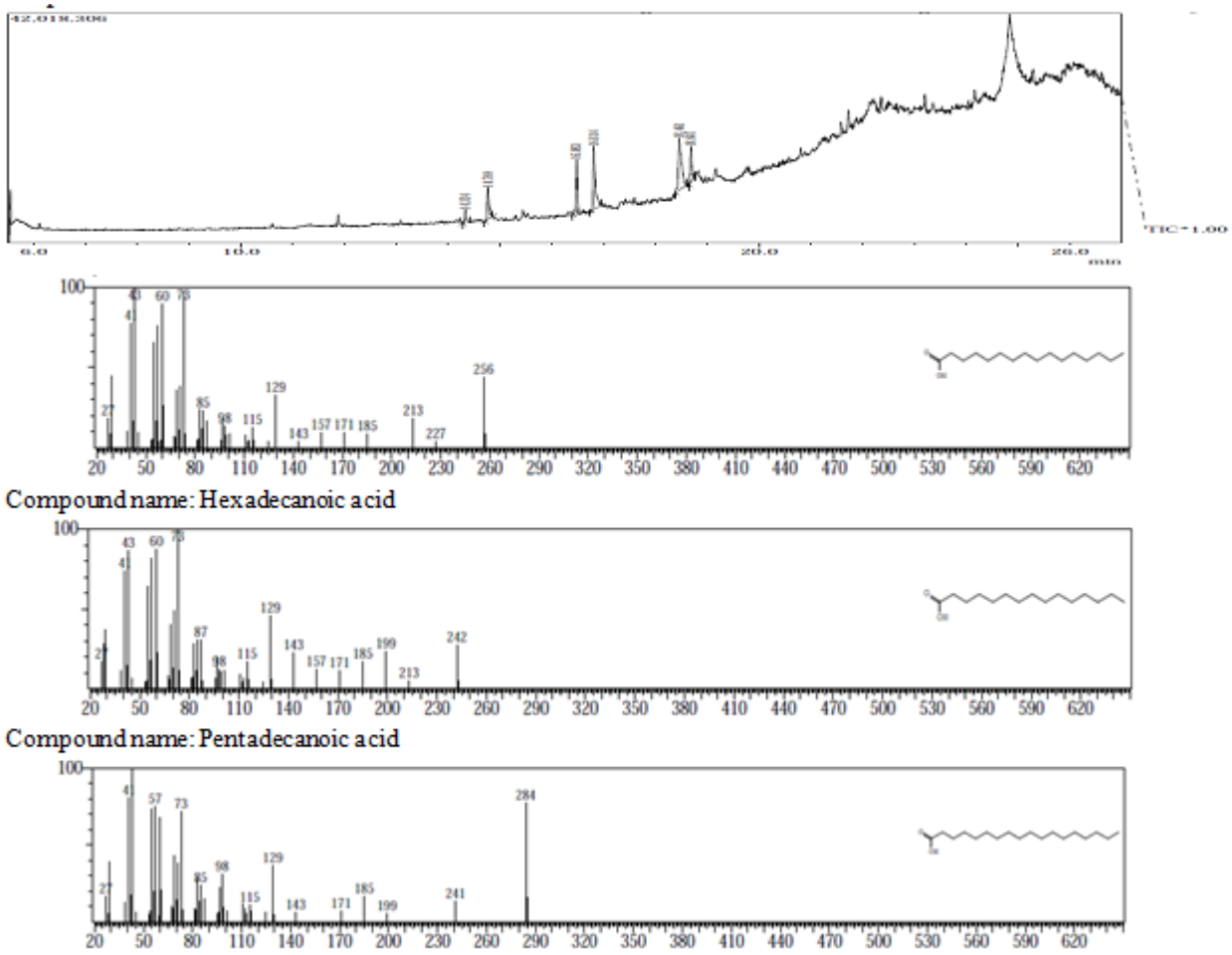
Retention Time: 3.575 min
Concentration: 10 µg/ ml
Area: 203815

	Analyte A:	Analyte B:
	2.392 min	3.842 min
	200 µg/ ml	200 µg/ ml
	240591	381906

The HPLC analysis shown one distinct peak in standard PHB (Fig.3.1) is 3.575 min and two distinct peaks in dye degrading consortium (Fig.3.2). One of analyte A shown peak at 2.392 min and other of Analyte B shown peak at 3.842 min retention time nearby to that of standard PHB.

Gas Chromatography – Mass Spectroscopy (GCMS)

Fig.5 PHA extracted from Bacterial Consortium grown in Nutrient broth with dye GCMS graph showing the peak of retention time



Thermogravimetric Analysis- Differential Scanning Calorimetry (DSC)

The thermogravymetric analysis results suggest that the PHB sample extracted from consortium is more thermostable than standard PHB. PHB extracted shown melting point was 222.4^{o}C where as standard PHB melting point was 218.48^{0} C (Figure6 A & B).

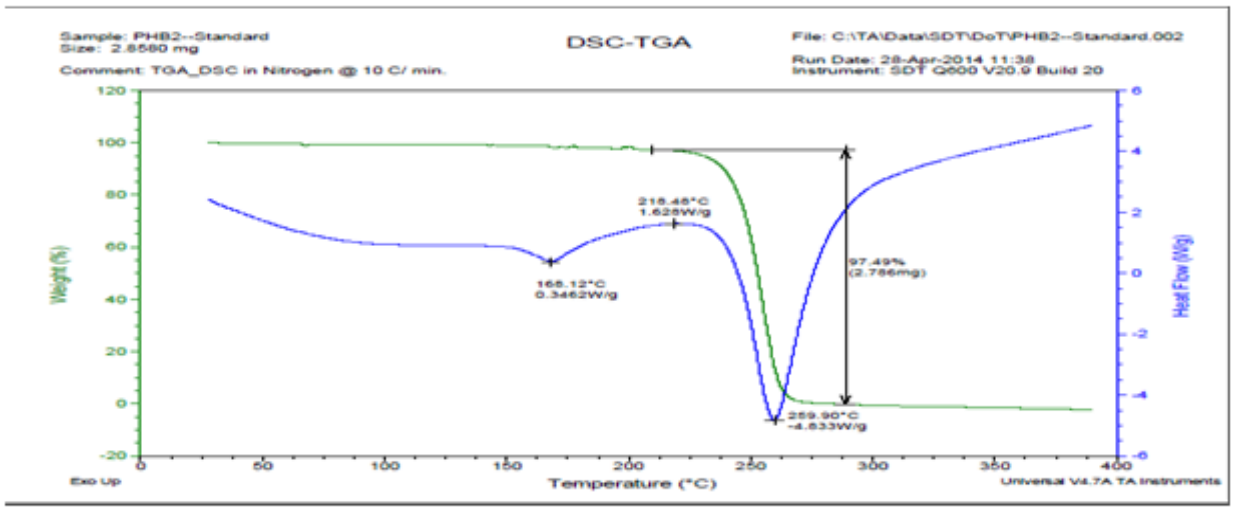


Fig. 6 A Thermo gravimetric analysis of standard PHB

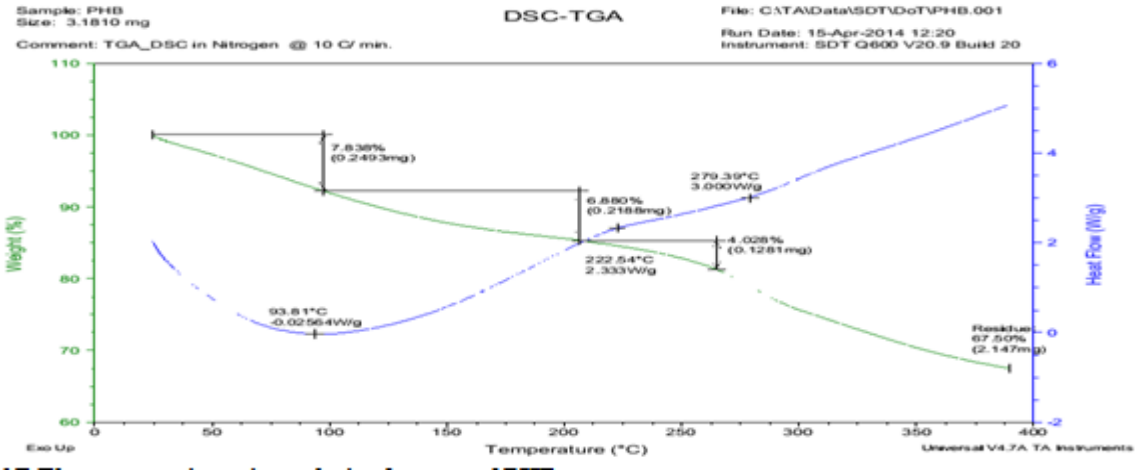


Fig.6 B Thermo gravimetric analysis of extracted PHB

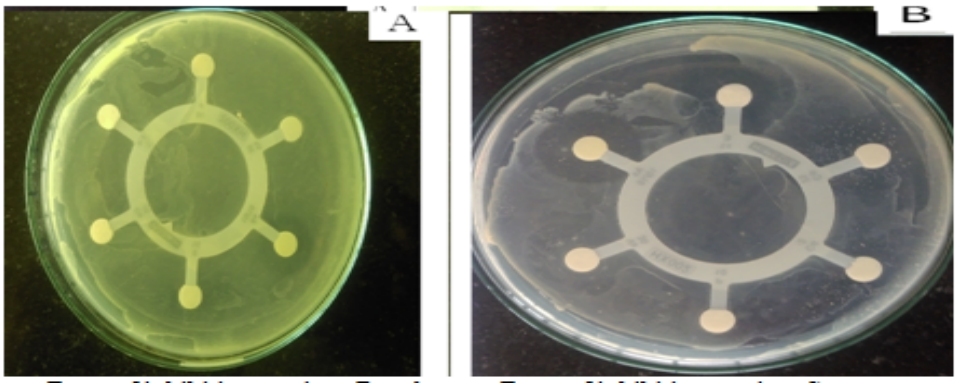
3.5 Antimicrobial property of biopolymer layered composites

Zone of inhibition was observed around the microbial polymer layered composites disc of 10 mg on *E. coli* and *S. Aureus* culture in nutrient agar plate. The disc of 1centimeter diameter was prepared from the biopolymer of bio-nano composites showed a zone of clearance on both plates containing *E.coli* and *S. aureus.*

Table5 Antimicrobial activity biopolymer layered silicate

Sr. No.	Biopolymer Layered Nanosilicate (PLS) of various concentration of MMT	Zone of Inhibition(cms)	
		E.coli	*S. aureus*
1.	0.5g	3.12 ± 0.065	3.36 ± 0.058
2.	1.5g	1.2567 ± 0.0309	1.1967 ± 0.1266
3.	2.0g	1.428 ± 0.0537	1.55 ± 0.0816
4.	2.5g	1.45 ± 0.171	1.42 ± 0.1257

Values are means of three sets ± SEM



Zone of inhibition against *E. coli* Zone of inhibition against *S. aureus*

Fig.7.1 A & B Antimicrobial activity of standard antibiotics agains *E. coli* and *S. aureus*

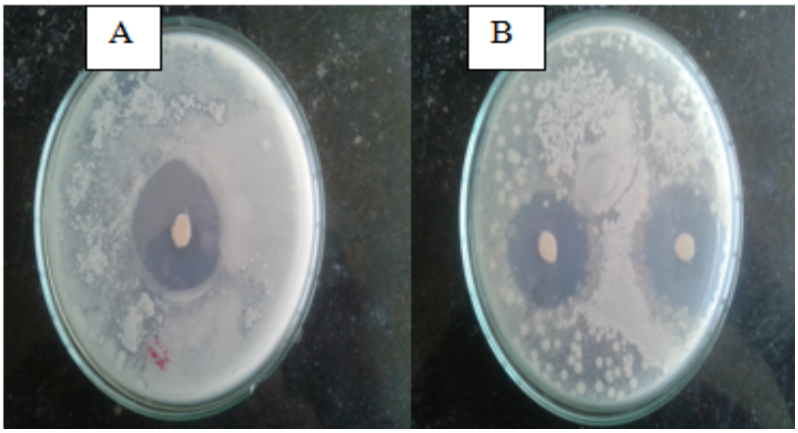


Fig 7.2: With bio composite A] the zone of clearance for *E.coli* B] The zone of clearance for *S. aureus*

Fig. 8 PHB Biopolymer layered composites spread and dried in Petri dish

IV DISCUSSION

Various textile dyes degrading biomass of *Providencia spp. RMG1, Bacillus spp.RMGII* and their consortium was efficiently produce PHA biopolymer from whey waste and molasses (Table 01).PHA synthase activity was more for the same waste as compare to other waste materials used for PS, BS and PB1 (Table 02).Decolourization of effluent, dry weight of biomass and PHA production were observed more for consortium as compare to the individual microorganisms of the consortium (Table 03).

PHB production from *Bacillus spp. RMGII* was 1.05gm/lit as compare to consortium it is 50% more. So, further process were proceeded by using PHB produced by using *Bacillus spp. RMGII.*Also after every consecutive batch the percent recovery was improved to 30%. The production of PHB concentration of acrylic acid was optimized for Bacillus *spp. RMGII* and *Provedentia spp. RMGI* and their Consortium.

PHB extracted from whey waste was 0.12 gm w/w of bacterial biomass. Acrylic acid concentration above the optimum level is inhibitory for microbial growth [17]. Therefore acrylic acid concentration was optimized in a range of 10µl/lit to 250µl/lit. At 37 ^{0}C, pH 4 and 7.5µl/50ml concentration of acrylic acid maximum production of PHB i.e. 51.95% by *B. spp.RMG II* was observed.

The concentration of PHB in grams was calculated as 1.12 gms /100ml (Table 04).The production of PHB was also observed through electron transfer system. It was observed that production increased slightly than the normal fermentation process.

FTIR analysis of the extracted biopolymer shown OH alcohol or OH carboxyl polymeric at 3456.49 cm^{-1}, Stretching sp3 for CH_2 and CH_3 at 2925.19 cm^{-1}, Carbonyl C==C-C==O (group of keto enolic) 1732.98 cm^{-1},$(C == C)_n$ group of polyena of PHA polymer 1458.71 cm^{-1},and supported with stretching C-O at 971.56 and 937.78 cm^{-1}[18](Fig.1 &2).

Thermogravimetric Analysis by using Differential Scanning Calorimetry (DSC) shows slightly more thermo stability with PHB extracted from dye degraded microbial biomass of consortium which makes it a good alternative for thremostable plastic.

HPLC analysis shown that standard PHB shown peak at one of Analyte A and other of Analyte B. Analyte A is having retention time far away from that of standard PHB whereas Analyte B is showing retention time nearby to that of standard PHB (Fig.3.1 & 3.2).GCMS graph shown PHB biopolymer was the derivative of Penta, Hexa and octa decanoic acid(Fig.5).

Antimicrobial activity of bio-composite was analysed. The zone of clearance was measured which was found to be 3.2 cm for 0.5g of MMT showed zone of clearance for *E.coli* whereas 3cm and 3.3cm zone of clearance for *S. Aureus* (Fig.7.1 & 7.2). The PLS for various concentration of MMT and PHB (1.5g and 2.5g) a distinct zone of inhibition of comparatively less diameter were observed. It was 1cm in diameter for 1.5g and 1.4 cm for 2.5g (Table 05).

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