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Polyhydroxybutyrate production accompanied by the effective reduction of chemical oxygen demand (COD) and biological oxygen demand (BOD) from industrial effluent

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Abstract−Industrial effluents are major pollution-causing agents for our environment. Our study focuses on utilizing effluents from different industries for efficient production of Polyhydroxybutyrate (PHB). Presence of PHB was identified by Sudan Black staining method. The PHB production parameters for *Pseudomonas aeruginosa* MTCC 4673 were studied critically, and it was found that glucose with 8.5 mg/L (0.0550 g PHB/g substrate) PHB concentration yielded the highest among the carbon sources used. Peptone with 8.9 mg/L (0.0524 g PHB/g substrate) of PHB concentration, an incubation period of 48 h and at a pH of 7 yielded the optimum results. These studies were compared with those of *Alcaligens latus* MTCC 2311. Dairy effluents (DE) and tannery effluents (TE) were considered for the best possible substrate, for the production of PHB in an optimized media. The results indicated that the dairy effluents gave a higher yield of PHB. Amongst various dilution levels studied from 10-100% (v/v), 50% (v/v) concentration of the dairy effluent showed maximum PHB productivity of 0.0582 g PHB/g substrate. A comparison of the chemical oxygen demand (COD) and biological oxygen demand (BOD) from the results, showed a significant removal percentage of 78.97% BOD and 53.482% COD, which highlighted the importance of utilizing effluents for PHB production, in order to reduce the risk of toxic effluent discharge. FT-IR analysis was carried out to confirm the presence of PHB.

Key words: Industrial Effluents, Sudan Black, Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD)

INTRODUCTION

The use of non-biodegradable synthetic polymer is a major threat to the environment. These synthetic polymers are xenobiotic, and hence, are recalcitrant to microbial degradation [1]. In response to the harmful effects of plastics, there has been a considerable interest in the development of biodegradable plastics [2]. Polyhydroxyalkanoates (PHA) are used as substitutes for plastics, as they have properties similar to those of various thermoplastics and elastomers, and also they are biodegradable in various environments [3,4]. Numerous bacteria synthesize and accumulate polyhydroxyalkanoates as carbon and energy storage material, under conditions of limiting nutrients, such as phosphorus, nitrogen, oxygen or magnesium in the presence of an excess carbon source [5].

Polyhydroxybutyrate (PHB) is the best known polyhydroxyalkanoate (PHA) [6]. PHB is a biodegradable thermoplastic with desirable properties, such as moisture resistance, piezoelectricity, and optical purity [7]. It is used in the production of biodegradable plastics, due to its similar structure to polypropylene [8]. PHB has ester linkages between hydroxyl butyrate, and therefore, it is easily degradable by both aerobic and anaerobic bacteria [9].

Under normal growth conditions, nutrient sources are used for the synthesis of proteins essential for the growth in bacteria. The nitrogen source depletion leads to the cessation of protein synthesis, which in turn leads to the inhibition of the TCA cycle enzymes, such as citrate synthase and isocitrate dehydrogenase, and consequently slows down the TCA cycle. As a result, the acetyl-CoA routes to PHB biosynthesis [10].

Bacteria that are used for the production of PHAs can be kept in two groups, depending on the culture conditions required for PHA synthesis. The first group, which includes *Alcaligenes eutrophus* [11], methylotrophs [12], and pseudomonads [13] and *Bacillus* spp., requires a limited nutrient medium in the presence of an excess of carbon source, for the efficient synthesis of PHAs. The second group, which includes *Alcaligenes latus*, *Azotobacter vinelandii*, and recombinant *Escherichia coli*, does not require any limitation of the essential nutrients for PHA synthesis. Since *A. eutrophus* accumulates a large amount of polymer in a simple glucose-salts medium, it has been used in research often for the production of poly (3-hydroxybutyrate) (PHB) [11]. *A. latus* has also been getting much attention towards PHA production, since it grows fast and accumulates PHA during growth without any nutrient limitation [14]. Since it can utilize simple carbon sources, cheap substrates such as raw sugar, beets, or cane molasses and industrial effluents can also be used. These advantages paved the way for a better development of the PHA production process by *A. latus*, utilizing industrial effluents.

P. aeuroginosa is one of the best known organisms for PHB production, and is able to produce PHB upto 60% on varying the temperature and oxygenation rates. PHB accumulating organisms are known to excrete PHB depolymerase, which under nutrient deficient conditions mobilizes intracellular PHB as a stored nutrient, to maintain metabolic functions.

PHB is used in the manufacture of packaging films, containers, and bags. It is also used as a biodegradable carrier for long term dosage of drugs, and medicines. PHB can blend with dendrimers to improve the mechanical and physical properties, which can be

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used for various applications.

The present study focuses on the production of PHB using *Pseudomonas aeruginosa* MTCC 4673 and *Alcaligenes latus* MTCC 2311 alongside with the effective removal efficiencies of chemical oxygen demand (COD) and biological oxygen demand (BOD), by utilizing raw industrial effluents. Studies on the effect of effluent dilution concentrations were also conducted, on the valuable production of PHB accompanied by COD and BOD removal.

MATERIALS AND METHODS

1. Microorganism and Culture Conditions

Pseudomonas aeruginosa MTCC 4673 and *Alcaligenes latus* MTCC 2311 were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, INDIA and stored in nutrient agar (NA) slants, which were stored at 4 °C.

A primary inoculum was prepared in a nutrient broth (NB) medium, containing (g/L): yeast extract, 0.5 g; NaCl, 1 g; peptone, 1 g; and incubated at 32 °C at 120 rpm for 48 h. *Alcaligenes latus* MTCC 2311 was used for comparing all the results that are obtained with *Pseudomonas aeruginosa* MTCC 4673.

2. Identification of PHB by Sudan Black Method

Sudan Black stain (0.3 g of Sudan black B powder added with 100 ml 70% ethyl alcohol) was used to reveal microbial intracellular polyhydroxybutyrate (PHB). Two drops of 0.7% Sudan Black solution were added to the heat fixed *P. aeruginosa* MTCC 4673 culture sample, which was then air dried and kept for 2 min. Safranine was added to the washed slide for counterstaining and dried for 10 seconds. The inclusion bodies were visualized at 100x magnification under a light microscope.

3. Optimization of PHB Production Parameters

3-1. Effect of Incubation Time on PHB Production

P. aeruginosa MTCC 4673 was inoculated to 100 ml of the production medium and kept for incubation at 37 °C. The experiment was carried out by maintaining the medium at different incubation time periods. For a fermentation period of four days, at various time intervals of 24, 48, 72, 96 h, sampling was done and it was subjected to the PHB quantification, using a UV-spectrophotometer at 235 nm.

3-2. Effect of Carbon Sources on PHB Production

A 24 h old *P. aeruginosa* MTCC 4673 inoculum was added to 100 ml of the production medium. The production medium in each flask was supplemented with different carbon sources (glucose, sucrose, lactose, and maltose - 20 g/L). The flasks were incubated at 150 rpm at 37 °C. The biomass and yield of PHB were quantified from the culture broth.

3-3. Effect of Nitrogen Sources on PHB Production

Production medium was added with different nitrogen sources (ammonium sulphate, peptone and tryptone). *P. aeruginosa* MTCC 4673 was then inoculated to each of the media, and kept at 37 °C for incubation. After the incubation time, the biomass and PHB quantifications were estimated from the cell broth.

4. Comparative Studies

4-1. Comparison of PHB Yields from Different Industrial Effluents as Carbon Source

The effect of various industrial effluents on PHB production was studied under optimized conditions. Raw effluents, like tannery effluent (TE) and dairy effluent (DE), were obtained from the industrial sites of Tamilnadu, INDIA, and their characteristics were studied (Tables 1 and 2). Both effluents were used in place of glucose as a source of carbon, inoculated with *P. aeruginosa* MTCC 4673 to the production medium. The initial characteristics of the raw dairy effluent (DE) and tannery effluent (TE) were determined before they were used in the production medium. The biomass and yield of PHB were determined after the incubation time. Results were compared with those of *A. latus* MTCC 2311 containing the production medium.

Table 1. Characteristics of dairy waste water

Parameter	Concentration (mg/L)
COD	1535
BOD	630
TSS	540
TKN	76
Total phosphorus	12
pH	7.3

Table 2. Characteristics of tannery waste water

Parameter	Concentration (mg/L)
COD	2380
BOD	1420
TSS	1035
TKN	120
Total phosphorus	12
Total phenolic compounds	28
Ammonium nitrogen	62
Total chromium	95
pH	8.4

4-2. Comparison of PHB Yields from Different Concentrations of Dairy Effluent

Varying concentrations of raw dairy effluent (DE) ranging from 10-100% were used as the major carbon source to study the effect of dilution of effluent on the production of PHB.

5. Study on COD and BOD Removal Efficiency

Since the chemical oxygen demand (COD) and biological oxygen demand (BOD) are the major factors in the physico-chemical parameters, they were taken as a reference for finding the effluent characteristics. The dairy effluent (DE) was sterilized and used as an alternative carbon source in the production medium, and incubated at 37 °C in a shaker (150 rpm). The biomass (g/L), PHB concentration (mg/L) and PHB yield (g PHB/g substrate) were quantified and extracted after the incubation time. After the extraction of PHB, the initial (raw effluent) and final (from PHB production medium) samples were collected and examined for the chemical oxygen demand (COD), biological oxygen demand (BOD) concentration levels.

5-1. Extraction of PHB

After the incubation period, the PHB produced was extracted chemically by the sodium hypochlorite method. The cells were harvested by centrifugation at 8,000 rpm for 10 min, and the pellet obtained was digested with sodium hypochlorite at 37 °C for 2 h. Cen-

trifugation was done with the digested sample, and the resulting pellet was washed thrice with ethanol, water and acetone, followed by the precipitation of PHB with chloroform. It was then allowed to evaporate at 60 °C to get crude PHB crystals.

5-2. Quantification of PHB

The amount of PHB in the extracted samples was determined spectrophotometrically at 235 nm, by the method adopted by Law and Slepcky (1960). For that, the polymer was dissolved in boiling chloroform and kept for 10 min. Then, 4.5 ml of concentrated H_2SO_4 was added to 0.5 ml of the chloroform-PHB mixture, and heated for 40 min at 100 °C in a water bath. The resultant crotonic acid was measured at 235 nm against H_2SO_4 as the blank.

The content of PHB in the cells was calculated as the ratio of the weight of the extracted PHB to the total cell weight, from which the PHB was extracted.

5-3. FT-IR Analysis

Ten mg of the extracted sample of PHB was subjected to the infrared (IR) analysis. The relative intensity of the transmitted light energy was measured against the wavelength of absorption on the region ranges from 400-4,000 cm^{-1} using a JEOL-FT IR-4000 plus double beam spectrometer. An IR spectrum of the sample was measured at ambient conditions.

RESULTS AND DISCUSSION

1. Identification of PHB

The presence of PHB was identified with the Sudan Black staining method. PHB was seen as black granules present inside the cell wall, counterstained pink with safranine. This confirmed the presence of PHB (Fig. 1) [15].

2. Effect of Time on PHB Production

The PHB yield and PHB concentration of 0.0494 g PHB/g substrate and 8.4 mg/L were obtained after 48 h of incubation; then, there was a sharp decrease in the yield (Fig. 2). The decrease in PHB accumulation observed beyond 48 h was attributed to the intracellular consumption of PHB, as the energy and carbon source [16-18].

3. Effect of Carbon Sources on PHB Production

The effect of various carbon sources on PHB production was studied, using *P. aeruginosa* MTCC 4673; it is presented in Fig. 3. The results showed that the PHB yield was the highest, when glucose used as a carbon source with 0.055 g PHB/g substrate PHB yield and 8.5 mg/L of PHB concentrations were obtained. Followed by glucose, sucrose yielded 5.1 mg/L of PHB concentration, lactose yielded 0.3 mg/L of PHB concentration, and maltose (0.4 mg/L of PHB concentration) was the least. Glucose was the preferred car-

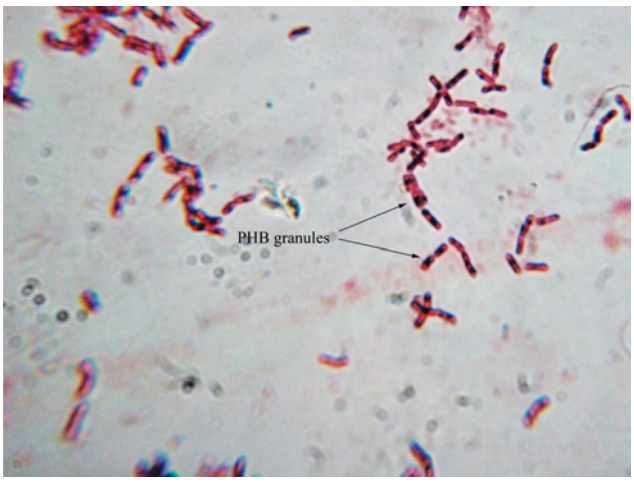

Fig. 1. Identification of PHB by Sudan Black Method at 100 x magnification.

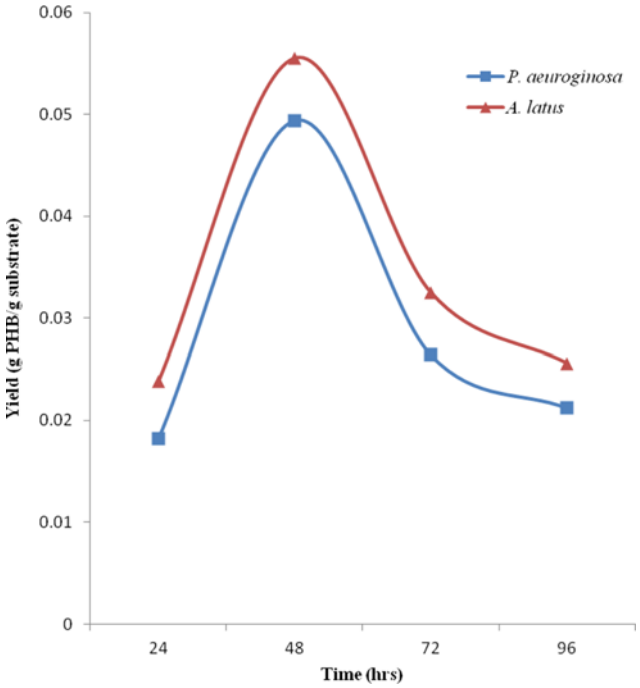

Fig. 2. Effect of different time intervals of fermentation on the production of PHB.

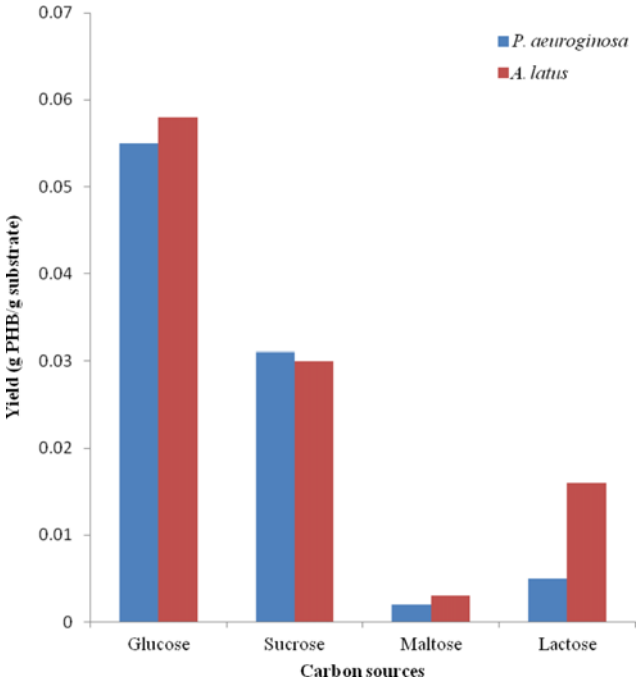

Fig. 3. Effect of carbon sources on the production of PHB.

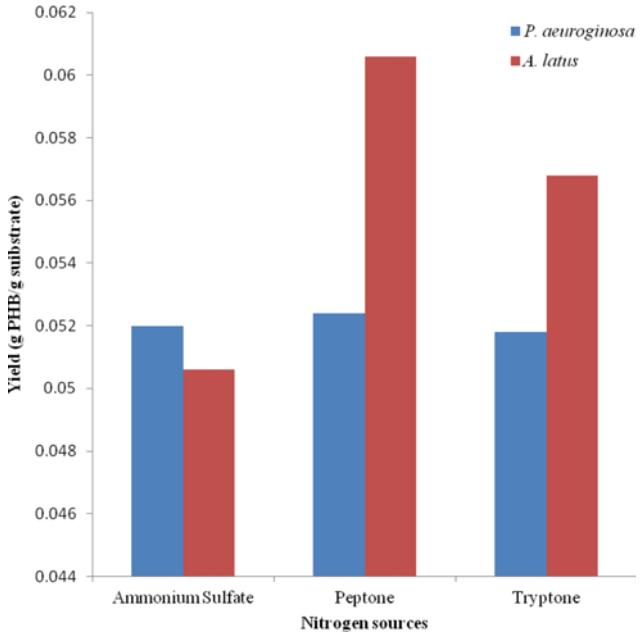

Fig. 4. Effect of nitrogen sources on the production of PHB.

bon source, and was taken for the other studies. The importance of carbon and nitrogen sources has been emphasized for the production of PHB. For example, the PHB production of *Alcaligenes eutrophus* was more in the medium using 1% glucose as the carbon source than in the medium using 1% maltose or sucrose [19].

4. Effect of Nitrogen Source on PHB Production

The influence of different nitrogen sources on PHB production is illustrated in Fig. 4. *P. aeruginosa* MTCC 4673 was able to utilize ammonium sulphate, peptone and tryptone as nitrogen sources for PHB production. However, peptone was the most effective nitrogen source with 0.0524 g PHB/g substrate PHB yield and 8.9 mg/L of PHB concentration. Peptone positively influenced the growth and PHB production, as it is needed by micro-organisms to synthesize all the enzymes that are directly involved, and to induce metabolic processes in a cell [20].

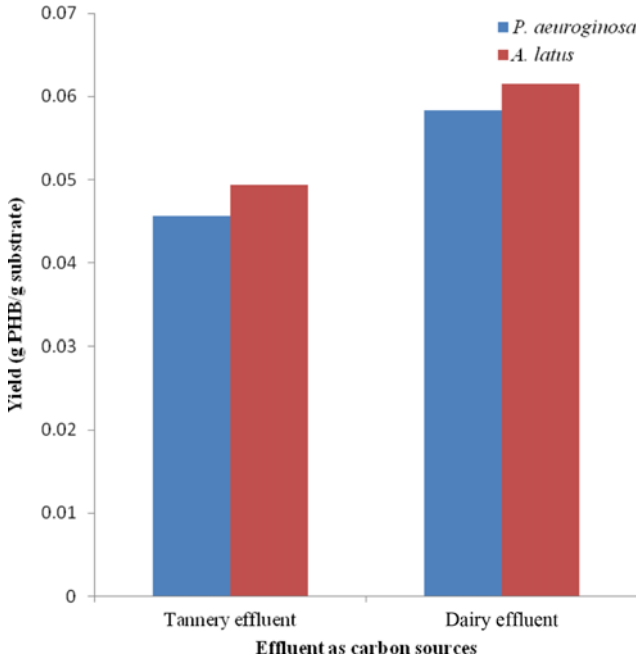

Fig. 5. Effect of effluent used as carbon source on the production of PHB.

Table 3. Effect of dilution of dairy effluent on PHB yield

Concentration of dairy effluent (%)	PHB concentration (mg/L)	PHB yield (g PHB/g substrate)
10	3.9	0.0234
20	4.3	0.0303
30	4.8	0.0489
40	5.4	0.0535
50	5.8	0.0582
60	6.1	0.0567
70	4.5	0.0376
80	4.4	0.0331
90	4.1	0.0248
100	3.5	0.0164

5. Effect of Industrial Effluents on PHB Production

The results from the use of dairy and tannery effluents as the carbon source for the PHB production are illustrated in Fig. 5. The PHB yield was higher in the medium supplemented with the dairy effluent (0.05831 g PHB/g substrate for *P. aeruginosa* and 0.06148 g PHB/g substrate for *A. latus*) when compared with the tannery effluent (0.0456 g PHB/g substrate for *P. aeruginosa* and 0.049412 g PHB/g substrate for *A. latus*) from the leather industry. The effect of various waste waters (dairy waste, rice bran and sea water) on the production of PHB was determined, and high results were obtained in the medium supplemented with dairy effluent [21].

6. Effect of Dilution of Dairy Effluent on PHB Yield

Table 3 shows the varying PHB yields at varying concentrations of the dairy effluent. Of the various concentrations used, 50% (v/v) concentration of the dairy effluent yielded the maximum PHB concentration and PHB yield of 5.8 mg/L and 0.0582 g PHB/g substrate, due to the availability of a very high organic content, and the low phosphate content which provided the ideal conditions for the production of PHB. The phosphate limitation decreases the ATP

Table 4. Effect of COD, BOD removal on different dairy effluent concentrations

Concentration of dairy effluent (%)	COD removal (%)	BOD removal (%)
10	19.34	44.87
20	25	48.07
30	27.615	56.41
40	35.074	63.67
50	53.482	78.97
60	46.588	54.48
70	38.98	42.85
80	33.75	30.76
90	30.87	29.2
100	23.81	25.64

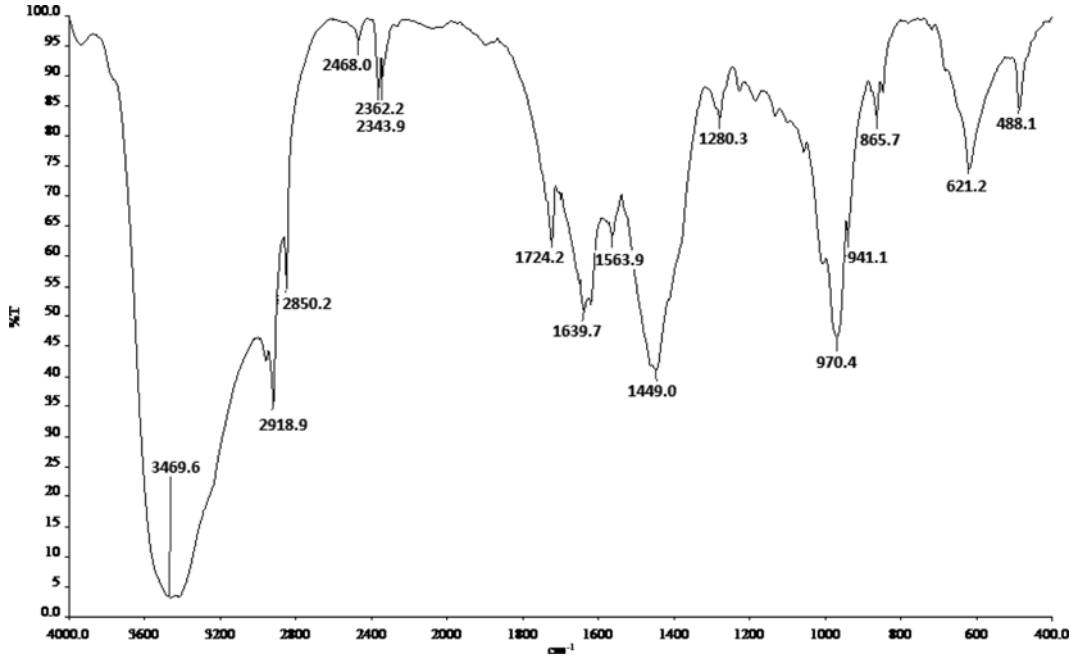

Fig. 6. FT-IR analysis of PHB extracted from *P. aeuroginosa* MTCC 4673.

production, which in turn, suppresses the TCA cycle enzymes, which in turn converts the available acetyl CoA into PHB [22]. The yield of PHB was reduced, when a higher concentration (>50%) of dairy effluent was utilized, due to the high content of the effluent components that overwhelmed the utilization of the nutrients. The dilution of various industrial effluents like dairy waste and sea water, and biodiesel wastewater [23] influenced the production of PHB.

7. Effect of Dilution of Dairy Effluent on COD and BOD Removal

The COD and BOD removal percentage is tabulated in Table 4. COD removal was found in range of 19% to 47.1%, and BOD removal was found in the range 25% to 80%. The highest BOD and COD removal percentage of 78.97% and 53.482% was obtained at 50% concentration of the effluent for the production of PHB. Comparing the results obtained from Fig. 5 and 6, the removal of COD and BOD increased with increasing yield of PHB. The optimal concentrations of T-N and T-P were 42 mg/L and 10 mg/L, and the maximum concentration of PHB was 5.8 mg/L. The results obtained by Sangyoka et al. also showed the influence of cassava starch manufacturing waste water (CSW) on the PHB yield and COD removal [24].

8. FT-IR

The IR analysis of the extracted sample showed the presence of methyl ester groups, which confirmed the presence of PHB (Fig. 6). The IR spectrum of the compound was recorded in the range of 400-4,000 cm^{-1}, and it showed characteristic bands for the groups C-H, C=O and C-O. The C-H stretching vibrations of methyl and methylene were obtained at 2,850.2 cm^{-1} and 2,918.9 cm^{-1}. The carbonyl group (C=O) gave a strong band in the region of 1,639.7 cm^{-1}. This frequency value was lower than the normal values because of polymerization. The C-O group showed a strong absorption band at 1,058.5 cm^{-1}.

The IR spectra of the PHA film and the purified methyl ester are identical [25]. The IR analysis of the extracted polymer was compared with the standard PHB chart as well as with that of the references [26].

CONCLUSION

The most preferred culture condition for *P.aeuroginosa* MTCC 4673 was studied to find the best possible way to produce PHB. *A. latus* MTCC 2311 was also used to compare the efficiency of the obtained PHB by *P. aeruginosa* MTCC 4673. Higher yield of PHB was recorded at 50% (v/v) concentration of dairy effluent with 0.0582 g PHB/g substrate with better removal efficiency of COD and BOD. The obtained results indicated the possibility of utilizing dairy effluent (sterilized raw effluent) as an alternative carbon source for an efficient yield of PHB. A novel approach of COD, and BOD removal efficiencies was also examined, that has proved to reduce their concentration levels while used in the production medium maintained for PHB production, so as to greatly reduce the environmental effects. Further studies may be conducted to enhance the large scale production of PHB at low cost, and also on discharging the less harmful effluents after utilization.

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