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Research Paper

Production of biopolymer from dairy waste: An approach to alternate synthetic plastic

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Abstract

Plastics are inevitable part of our modern life and used in different sectors of society like packaging, consumer products and many more. Synthetic polymers are almost not degradable by natural processes in the environment. Present work illustrates an effort to isolate bio-polymer producing bacterial strains from dairy industrial influents. Ten bacterial colonies were isolated which were stained using Sudan Black B dye in order to test their ability to produce bio polymer like granules. Out of ten, five strains were found to produce bio-polymer like granules. These fives isolates were further grown at different pH, incubation periods, salt concentration and different carbon sources to know the optimum growth and production. Bio-polymer like granules was extracted by sodium hypochlorite chloroform method and estimated the accumulation of granules. Two strains were found with highest bio-polymer accumulation when the buttermilk was used as one of the cheaper carbon sources. The study shows that PHB accumulation depends on residual biomass is inversely proportionate with the PHB accumulation in the cell.

Keywords: PHB, Bacteria, Strain, Colony, Granules.

Introduction

Rapid industrial development emits wide range of chemicals and materials used in the production process directly and most of them are harmful to the environment. Moreover non degradable polythene based plastic used in our day to day activities are unfriendly to the environment. Since synthetic plastics marked their first form in 1950s, they have emerged to be among the most needed material in our daily life. In harsh conditions petroleum based plastics are reportedly stable against chemical degradation and microbial decomposition, thus rendering them durable, highly resistant and persist for very long time in the environment[1]. Due to their excellent properties and wide range of application, synthetic plastics have ace the commodity market and helped in establishing technologies related to plastics manufacturing. Various types of wastes produced from agriculture industry, dairy industry, oil industry has turn into a major environmental issue[2]. These wastes contain useful ingredients for the development of Polyhydroxyalkanoates through microorganism. The polyhydroxyalkanoates (PHA) can be further converted in to Polyhydroxybutyrate (PHB) which is a biodegradable plastic raw material[3].

Polyhydroxyalkanoates (PHAs) are polyesters which are naturally synthesized and found to be accumulated in intra-cellular membrane of numerous bacteria as intracellular energy storage materials during unbalanced growth[4-7]. PHA is polyester of hydroxy fatty acids which is naturally produced by many bacteria as an intracellular carbon and energy reserve material[8].

The family of PHAs includes several polymeric esters such polyhydroxybutyrates, polyhydroxybutyrate co-hydroxyvalerates (PHBV), polyhydroxybutyrate co hydroxyhexanoate (PHBHx) and

polyhydroxybutyrate co-hydroxyoctonoate (PHBO). Poly 3-hydroxybutyric acid (PHB) is the most common natural microbial PHA. In some microbial species, accumulation of PHA occurs during the presence of excess carbon and a limitation of nitrogen sources[9]. Polyhydroxybutyrate (PHB) is a biodegradable and biocompatible thermoplastic, there are classes of bacterial polyesters collectively called polyhydroxyalkanoates (PHAs), accumulated intracellular as reserve granules by some bacteria in harsh environmental conditions[10]. Polyhydroxybutyrate (PHB) was first discovered by Lemoigne in 1926 in *Bacillus megaterium*[11]. PHB production cost is dependent on several factors like substrate, chosen strain, cultivation strategy and downstream processing[12].

Materials and Methods

Sample collection and isolation of pure cultures

Influent samples were collected in sterile bottle from two dairy industries of Central and North Gujarat. A serial dilution method was used for step wise dilution of the substance in solution. Serial dilution indicates that an identical volume of material is being transferred from one vessel to another. This procedure increases the dilution of a substance by certain increments. In 9 ml sterile distilled water, 1ml influent sample was dissolved and the sample was serial diluted in sterile distilled water. 10^{-1} to 10^{-5} dilution used for the isolation of bacterial strains. The plates were incubated at 37^{o}C temperature for 24 hours in inverted position in incubator[13].Colonies with different characteristic features were maintained as pure cultures on nutrients agar slants and stored at 4^{0}C.

Screening of PHB Producing isolates by Sudan Black B Staining

The isolated colonies were stained by Sudan black dye[14]. 0.3% Sudan stain was spread on a slide to form a smear and allowed it to dry for 15 minutes then dipped in xylem for 10 second. After spreading, safrenin was applied for 30 second and washed with water and left to dry. The PHB granules appeared as blue black droplets and cytoplasm part of microorganisms appeared as pink where as yellow-brown droplets shows the absence of PHB[15].

Characterization of PHB producing isolates

Only sudan positive strains were further identified and characterized by their morphological and biochemical characterization according to Bergey's Manual of Determinative Bacteriology[15].

Morphological Characterization

Morphological features were studied by growing the cultures on nutrients agar media and gram's staining.

Biochemical characterization

Different Biochemical tests were carried out including Methyl red, Vogues Procurer, Indol and H_2S production.

Optimization of PHB

The isolated strains were grown in carbon containing Modified Mineral Salt Basal (MMSB) medium at different pH like 2, 5, 7, 9 and 11 along with various salt concentration like 0.1%, 0.2%, 0.5%, 5%, and 10%. Growth curve study was checked with different carbon source like Buttermilk, glucose, Cotton oil and starch. Cultures were taken in different flasks for incubation periods of 24 hours. Later on optical density of different pH, salt concentration and different carbon source containing medium was observed in UV-visible Spectrophotometer at 600nm.

Fermentation of PHB

The PHB producing strains ware grown in carbon and nitrogen limiting mediums. Different types of carbon sources and nitrogen sources used for large scale industrial production of PHB. The nutrient broth containing microorganisms were 5µl culture inoculated in MSBB medium. Composition of MSBB media : In 250 ml of modified mineral salts basal medium (MMSB) contain: $(NH4)_2HPO_4$ (1.1g), K_2HPO_4 (3.7g), 10 ml of 0.1M $MgSO_4$ and 1.0 ml of a Microelement solution (this microelement solution contained $FeSO_4$-$7H_2O$ (2.78g), $MnCl_2$- $4H_2O$ (1.98g), $COSO_4$-$7H_2O$ (2.81g), $CaCl_2$- $2H_2O$ (1.67g), $CuCl_2$-$2H_2O$ (0.17g), and $ZnSO_4$-$7H_2O$ (0.29g) in 1 liter of 1 N HCl)[13]. The pH was adjusted to 7 and adds 1% (v/v) buttermilk as a carbon sources. The flasks were shaken in rotary shaker for about 72hrs at 150rpm. The microbial cultures were centrifuged at 5000 rpm for 15-20 minutes and the pellets were harvested and washed with distilled water. These pellets were dried by incubating them in oven at 80^{0} C temperatures for overnight.

Cell dry weight

The dry PHB granules were further incubated for 72Hrs for 15 min. The cell pellets were dried to estimate the dry cell weight (DCW) in units of g/ml.

Extraction and quantification of PHB

The pellets were quantified with 4% sodium hypochlorite solution at 37^0C for 20 min and were collected by centrifugation at 10,000 rpm for 15 min, washed with water, acetone, and ethanol respectively for washing and extraction. Finally polymer was dissolved in chloroform and kept in complete evaporated space[16].The dry weight of extracted PHB was estimated in g/ ml. The residual biomass was estimated as the difference between dry cell weight and dry weight of PHB. The percentage of intracellular PHB accumulation was estimated as the percentage composition of PHB present in the dry.

$$Residual\ Biomass\ (g/ml) = (\text{DCW (g/ml)} - \text{Dry Weight of extracted PHB (g/ml)}$$

$$PHB\ accumulation\ (\%) = \frac{\text{Dry weight of extracted PHB } \left(\frac{g}{ml}\right)}{\text{DCW } \left(\frac{g}{ml}\right)} \text{x100}$$

Results and Discussion

Isolation of microorganisms

Total of 10 bacterial colonies with different morphological features were isolated and their morphological characteristics are illustrated in table 1.

Morphological characteristics	Strin-1	Strain-2	Strain-3	Strin-4	Strain-5	Strain-6	Strain-7	Strain-8	Strain-9	Strain-10
Shape	Big (Irregular)	Small Round	Big Round	Big (Irregular)	Small Round	Irregular	Small Round	Small Round	Big (Round)	Small Round
Colour	Yellow	Pink	White	Creamy white	White	White	Creamy white	White	Creamy white	White
Elevation	Pulvinate	Convex	Convex	Flat	Raised	Flat	Raised	Raised	Convex	Raised
Margin	Entire	Entire	Entire	Entire	lobate	Repined	Erose	Entire	Undulate	Entire
Surface	Smooth	Smooth	Smooth	Smooth	Punctuate	Punctuate	Smooth	Smooth	Smooth	Punctuate
Consistency	Moist	Moist	Dewdrop	Moist	Dry	Dry	Dewdrop	Butyrous	Butyrous	Dry
Optical Characteristics	Opaque	Opaque	Porceleneous	Opaque	Opaque	Opaque	Subceous	Opaque	Opaque	Opaque

Table 2: Results of Biochemical tests of PHB producing strains

Biochemical Tests	Observations				
	Strain-1	Strain-2	Strain -3	Strain -7	Strain -8
Indole	Positive	Positive	Positive	Negative	Positive
Methely Red	Positive	Positive	Positive	Positive	Positive
VP	Positive	Negative	Positive	Negative	Negative
TSI	Positive	Negative	Negative	Positive	Negative

Screening of PHB Producing Bacteria

Out of ten colonies five showed positive results on Sudan Black B stain (figure 1).

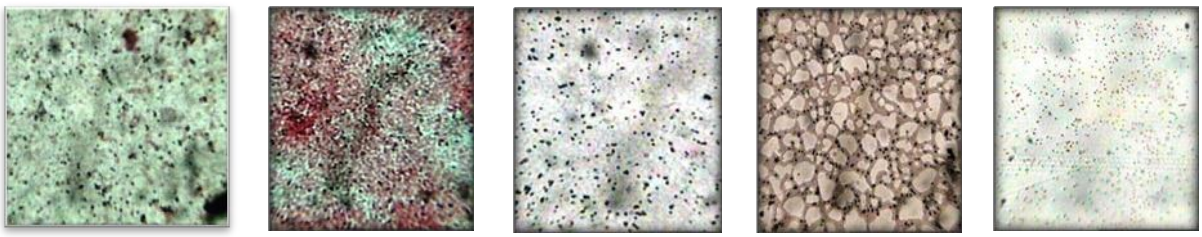

Figure 1: Strains 1, 2, 3, 7 and 8 showing PHB Granules with Sudan Black B Staining

Characterization of PHB producing isolates

Biochemical characteristics

Different biochemical tests have been performed for PHB producing strains. The methyl red was found positive with all isolates and strain 1 was positive for all tests. Table 2 shows the detail of biochemical tests perform for all the isolates.

Effect of pH on bacterial growth

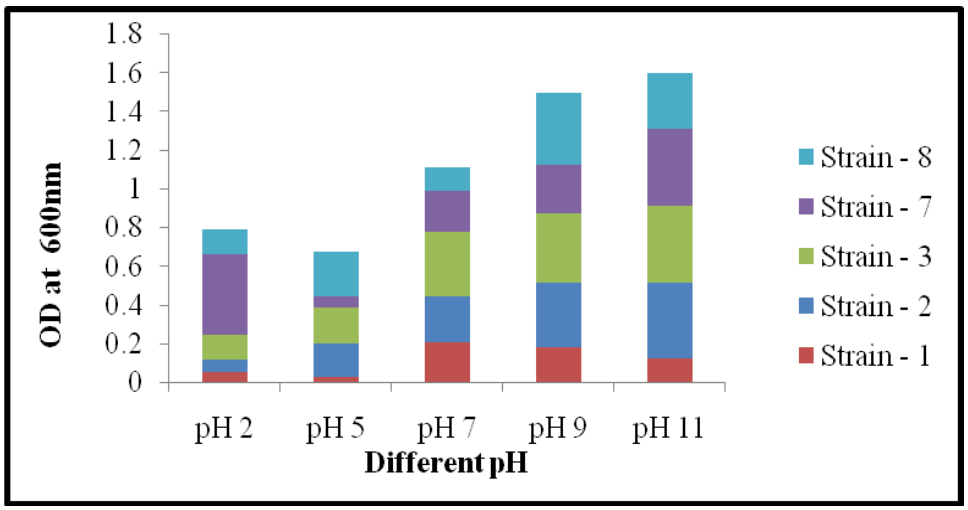

Figure 2: Effect of pH on bacterial growth

Figure 2 depict the cell growth at different pH. Most of the isolates show higher growth in alkaline pH while strain 2 grows in both acidic and alkaline medium. In present study pH 11 found to be optimum for bacterial growth.

Effect of salt concentration on bacterial growth

The isolates were grown in medium with different salt concentrations (from 0.10 to 10%). It was found that all the strains showed their growth at 0.2% salt concentration. It was further observed that different organisms show their growth performance at the concentration of 0.2%, where as strain 8, in present study perform maximum growth at 5% salt concentration.

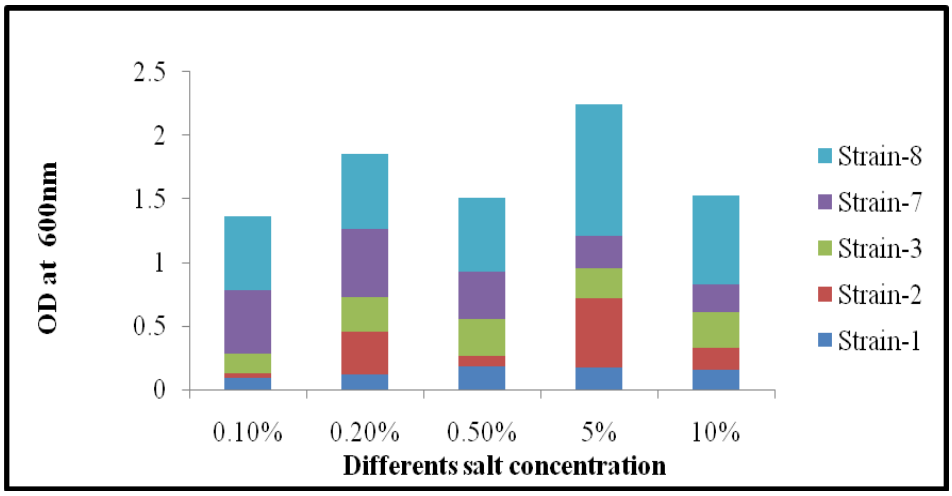

Figure 3: Effect of salt concentration on bacterial growth

Incubation period

The maximum growth was observed for all the isolates at 72 hrs incubation period. In present study, we have not performed the growth study after 72 hrs, however literature suggests that most PHB producing bacteria shows higher PHB production after 72 hrs[17].

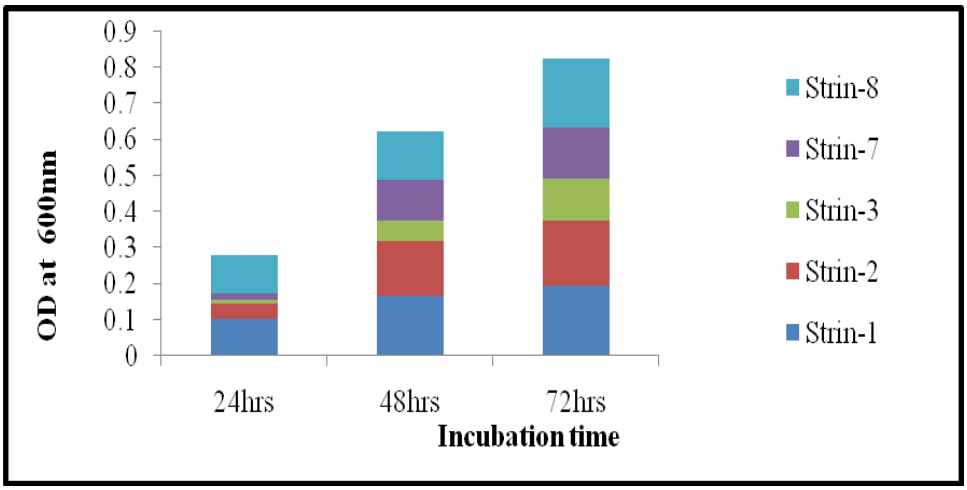

Figure 4: Incubation periods for PHB producing isolates

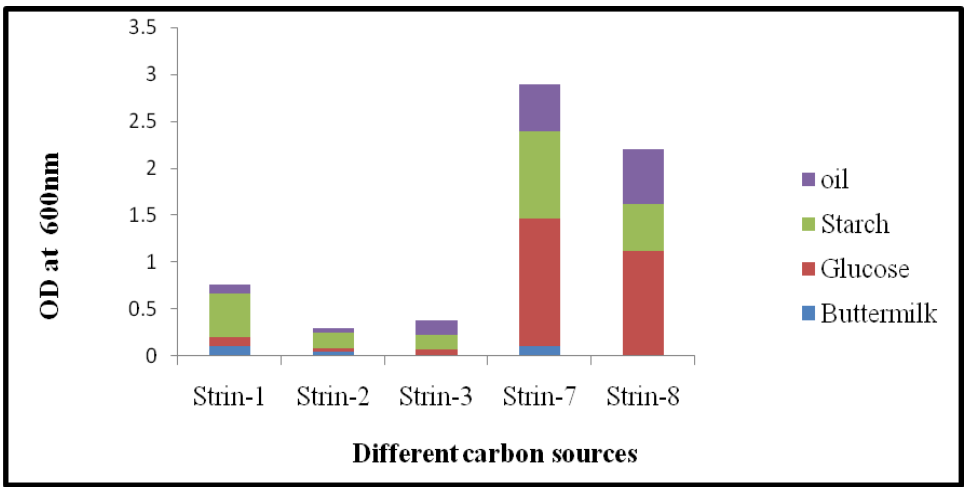

Figure 5: Effect of different carbon source

Effect of different carbon source on bacterial growth

Both glucose and starch are better carbon source for bacterial growth, but looking at the applicability and cost effectiveness, cheaper carbon source like buttermilk was tested. Figure 5 reveals that strain 7 shows significant growth when buttermilk was used as a carbon source, where as edible oil gives comparatively good growth of all the isolates. Several carbon sources were used by various researchers to increase cost effectiveness of cheap PHB production like, jiggery and Fructose by Dhingra (2012).

Extraction and quantification of bio-polymer

Isolates were grown in MMSB (modified mineral salts basal) medium for 7 pH and 72 hrs incubation time and rotated at 150 rpm with buttermilk as a carbon source. The extracted granules and their dry weight, residual biomass along with PHB accumulation from all the five isolates is illustrated in table 3.

Table 3: % Accumulation of PHB from granules

No	Cell dry weight (g/ml)	Dry weight of PHB (g/ml)	Residual biomass (g/ml)	% Accumulation of PHB
1	0.12	0.07	0.05	**58.33%**
2	0.8	0.04	0.76	**5%**
3	0.12	0.0	0.12	**0%**
7	0.11	0.03	0.08	**27.27%**
8	0.12	0.01	0.11	**8.33%**

The percentage of intra cellular PHB accumulation was estimated as the percentage present in the dry cell weight. it can be seen in table 3 that strain-1 show maximum PHB accumulation (58.33%) with 0.12 g/ml cell dry weight, followed by strain-7 showing 27.27% and 0.11 g/ml cell dry weight and where as strain 3 did not produced PHB even the cell dry weight was 0.12 g/ml. Above table also suggests that residual biomass is inversely proportionate with the PHB accumulation in the cell.

FTIR analysis of bio-polymer

Table 4 shows characteristic bends C-O, C = O, C=C and -OH alcohol gives strong bend in the range of 3554, 3478, 3415 and 3235 this frequency value were higher than the value of polymerization. The carbonyl group (C=O) provides strong band in the range of 1638 and 1618. The (C-O) group show strong and broad absorption 1300 – 1000. Table 4 depicts the comparison between PHB powder obtained from microorganisms and PHB Sigma which is available in market.

Table 4: FTIR Results

Comments	PHB Powder from present study	PHB (Sigma) (Marjadi 2011)
H bond	3415	3417.70
CH group	2926	2928
Carbonyl Group	1618	1673
Intramolecular H bond	3554	3330.13
Trans RCH=CHR	972	-
Ester Group	-	1076

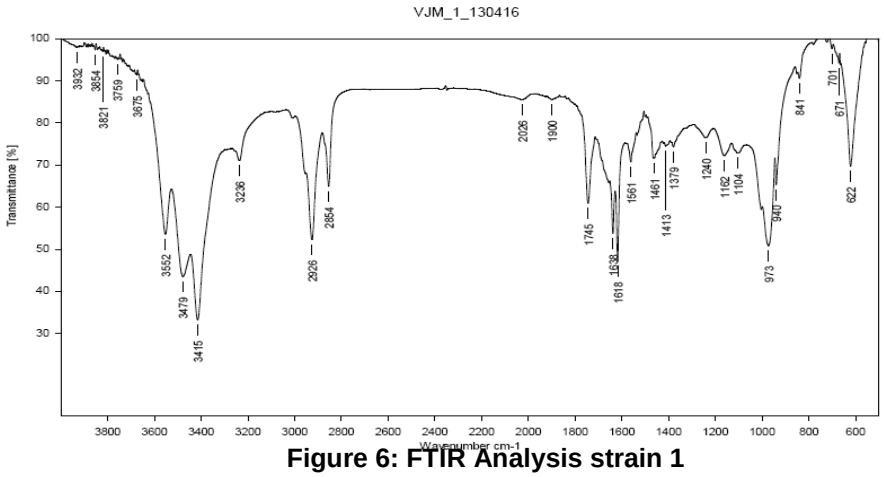

Figure 6: FTIR Analysis strain 1

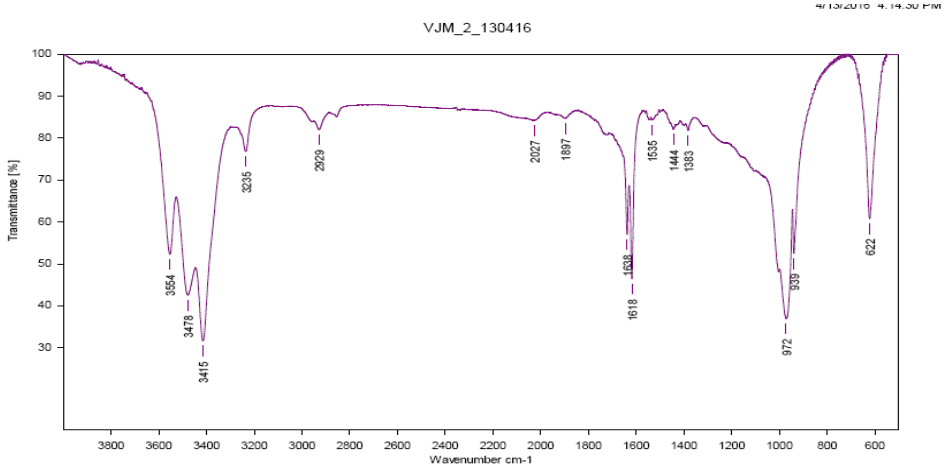

Figure 7: FTIR Analysis strain 7

The comparison of peaks between microbial PHB and standard PHB[13] reveals the similarity of PHB powder produced in present study with the standards (Table 4). Figure 6 and 7 shows the results of FTIR analysis carried out of strain-1 and strain -7 which were observed to produce comparatively high amount of bio polymer.

Conclusion

Total 10 microbial strains were isolated and identified from all the samples by analyzing their morphological characteristics. Out of which 5 were categorized as producers of bio-polymer as they provided positive results when stained with Sudan Black Staining. The optimization study revealed that, usage of buttermilk as a carbon source at pH 7, Temperature 37 ^{o}C, shaking speed of 150 rpm gives maximum production of bio-polymer. Moreover, Strain-1 was found with maximum PHB accumulation (58.33%) followed by 27.27% from Strain-7, whereas, Strain-3 did not shown any accumulated PHB granules. These granules were further confirmed for their PHB like nature through FTIR analysis. The research work suggests that the buttermilk can be the cheaper carbon source to produce microbial polymer similar to PHB from the microorganisms isolated from the dairy wastes.

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