IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)
e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 8, Issue 4 Ver. II (Apr. 2014), PP 26-31
www.iosrjournals.org

Poly-β-hydroxybutyrate (Bio-plastic) production utilizing Waste Effluent of a Sugar Industry

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Abstract: *Clarification of the molasses is an important step in the sugar industry. The waste effluent from the lime phospho-floatation method of clarifying molasses is subjected to a feasibility study as feedstock for bacterial production of poly-3-hydroxybutyrate P(3-HB) a well known bio- plastic. A chemical analysis of the waste effluent has been done to ascertain the total assimilable carbon content in terms of glucose and sucrose, along with the micronutrients like calcium, phosphates and ascorbic acid (vitamin C). Different proportions of waste effluents and inorganic mineral salts are tested in terms of P(3-HB) accumulation by the organism BP/SU1 of Staphylococcus epidermidis, origin. The production of P(3-HB) is optimized against the best utilization of waste effluent and biomass generation. The optimal proportion of waste effluent and inorganic mineral salts is upgraded to 1.5 litre bioreactor scale and the purity of the product thus obtained is confirmed.*

Keywords: *Bio-plastics, Staphylococcus sp, waste effluent, sugar industry, linear programming*.

I. Introduction

Polyhydroxyalkanoates, PHAs, have long since established themselves as thermoplasts or elastomers depending upon their composition [1]. In addition to their excellent polymeric properties PHAs are completely biodegradable and renewable [2]. With the discovery of R-3-hydroxy butyric acid and its low molecular weight polymers in human tissue and blood [3] poly-3-hydroxybutyrate P(3-HB), one of the most important polyhydroxyalkanoates, promises to become an excellent media for drug delivery and biomedical application in near future. Any attempt to make P(3-HB) economical should entail two considerations [4].

1. Lowering the cost of raw materials (which comprises about 30% of the total cost).
2. Lowering the processing cost (which comprises about 70% of the total cost).

The first aspect of the problem could be solved by utilization of any appropriate waste stream of an agro-industry as a feedstock for the biotechnological process. Selection of the waste stream should be made in such a way that it could be suitably integrated into the global region where the production plant will be constructed [5]. Since sugar industry is a suitable component of the Indian economy, the waste effluent of sugar industry could be explored as a lucrative option towards becoming the substrate for bacterial production of polyhydroxyalkanoate [6]. If this effluent could be harnessed to act as a nutrient media for the polyhydroxybutyrate accumulating microorganisms, not only the production expenses of P (3-HB) would be substantially lowered, the sugar industry will be provided with a strategy to overcome disposal problems of an effluent with high Biological Oxygen Demand (BOD) [7]. Keeping in view the above mentioned objectives, the waste effluent of a sugar refinery was explored as viable substrate for the production of P(3-HB) by biomass assimilation of the P(3-HB) accumulating organism BP/SU1 of the species *Staphylococcus epidermidis* as shown in our previous paper [8].

II. Materials and Method

1.1 Sample collection:

Shree Renuka Sugar Mills, a sugar refinery industry situated at Debhog, Haldia (co-ordinates 22˚.03′N, 88˚.06′E) in West Bengal is chosen as the model system to explore the feasibility of converting waste effluent into biomass for polyhydroxybutyrate production. The liquid waste effluent of Shree Renuka sugar mills was analyzed for its Ca^{+2} ion content by complexometric titration using ethylene diamine tetra acetic acid EDTA as chelating agent. Its phosphate content was assessed by spectro-photometric method using molybdenum blue generation [9]. The sugar content, in terms of both glucose and sucrose was estimated by Fehling's method and spectro-photometric method using anthrone reagent [10]. Vitamin C (ascorbic acid) is estimated iodometrically. All experiments for estimation were done in triplicate and the mean value has been noted. BOD (Biological oxygen demand) and COD (Chemical Oxygen demand) is measured using standard protocols.

1.2 Standardization of growth of BP/SU1 using the waste stream of sugar plant

WE (Waste Effluent) of sugar plant and mineral media [11] consisting of inorganic mineral salts (components of mineral salt media per litre of solution KH_2PO_4:2g, K_2HPO_4:7g, NH_4NO_3:1g, $MgSO_4.7H_2O$:0.1g, $ZnSO_4.7H_2O$:0.001g, $CuSO_4.5H_2O$:0.0001g, $FeSO_4.7H_2O$:0.01g, $MnSO_4.6H_2O$:0.002g) only are mixed in different proportions to produce the culture media of varying nutrient concentrations ranging from 0% WE to 100% WE. The mixtures are autoclaved at 15 P.S.I for 15 minutes for complete sterilizations. They are then inoculated with 1% BP/SU1 culture grown in enriched media for 16 hours and incubated for 48 hours in shake flask condition at 37°C. The suitability of these different mixtures as a culture media for BP/ SU1 growth is measured in terms of the OD at 600 nm and cell dry weight (CDW) for 48 hr cultures using the same mixture of waste effluent and mineral salts maintained under identical conditions as blank. The accumulation of P(3-HB) is ascertained qualitatively by Nile blue A staining [12] and assayed by the method of Law and Slepecky [13]. A fixed volume of cell suspension is centrifuged at 6000 rpm for 15 minutes at 4°C and the cell pellet is dried under vacuum until constant weight is obtained, to get the cell dry weight.

1.3 Isolation and purification of the P(3-HB) obtained from the waste effluent

The mixture which gives the optimized production of P(3-HB) is used as the feedstock for the 1.5 litre culture in MBF250 ME fermentor (Eyela, Japan), pH is maintained at 7.0, temperature kept at 35°C, aeration at 0.075 MPa, and the growth is continued for 48 hours before harvesting . The harvested cells are subjected to chloroform – sodium hypochlorite treatment for P(3-HB) extraction. In this method the P(3-HB) is extracted according to the traditional method of Law and Slepecky by sodium hypochlorite followed by boiling with chloroform under reflux and filtration. The filtrate is evaporated in a vacuum chamber to get ultra pure P(3-HB) . The purity of the extracted P(3-HB) is judged by crotonic acid assay against standard P(3-HB).The waste effluent used as a nutrient media has its BOD ,COD and total carbon content determined both after and before the bacteria grows in it to ascertain the carbon uptake by the organism during the process.

III. Results and discussion

From Figure (1a) it is evident that

- The raw material for this industry is molasses which is obtained from local villages by primary evaporation of sugar cane juice.
- There are two types of waste generated in this sugar industry. One is the liquid scum which comes out of the raw melt after the lime and phosphoric acid aided clarification process and the other is solid particles which have been trapped into the scum and removed by filtration.

The liquid scum passes through the clarification process till its sucrose content has dropped significantly and it is no longer industrially viable to extract sugar from it by running it through the costly centrifugation, evaporation and decolourization processes. The solid waste is left to dry and ultimately sold to the nearby villagers as a fodder additive with the bagasse which has been generated during sugarcane crushing. The liquid waste is just allowed to run down in a cess pool adjoining the sugar refinery plant (Figure 1c). This cess pool with its potentially high Biological Oxygen Demand (about 40-60,000 ppm) classifies as a microbial pollutant and has to be taken care off, by expensive disposal techniques adhering to strict environmental rules. We have experimented with this waste effluent (WE) as our feedstock to breed the P(3-HB) accumulating organism BP/SU1 *Staphylococcus epidermidis*.

Complex waste streams contain additional substances like minerals and vitamins that provide the production strain in bioprocesses with the micronutrients that have positive impact on the bacterial cultivation [14]. Table 1 shows the content of the various chemicals present in the WE. Each experiment has been done in triplicate and mean value has been recorded. From the experimental results it is evident that the spent flow of the industry has enough chemical nutrients to sustain the growth of BP/SU1, from which P(3-HB) will be obtained.

Staphylococcus epidermidis strain BP/ SU1, MTCC accession No.9538 [15] can accumulate substantial amount of polyhydroxybutyrate. However tryptone and yeast extract are expensive nutrient media, hence different proportions of WE has been mixed with low cost dilute solutions of inorganic salts to replace them. It has been found out that with laboratory grade chemicals the cost of the mineral salt media is only INR 11.26 per liter, excluding the cost of autoclaving and distilling the water, as distillation may be avoided. Figure (2) depicts this cost factor as a function of the different concentration of WE in the feedstock used to generate P(3-HB) production. P(3-HB) production can be viewed as a function of three inter related parameters. These are the values of OD at 600 nm which gives an idea of the amount of cell accumulated, the cell dry weight gives a measure of the amount of biomass generated and the direct quantitative estimation of P(3-HB) by oxidizing it to crotonic acid and measuring the OD at 235 nm. The P(3-HB) accumulation is also verified qualitatively by fluorescence microscopy of Nile blue A stained cells as shown by Figure (3).

From Figure (2) we can see the amount of cell accumulated is a linear function of the WE [16] in the solution .In order to lower the cost of the feedstock and yet get the maximum amount of P(3-HB) production, linear programming is used to generate basic feasible solutions in form of polytopes. Among these basic feasible solutions [17] (vertices of polytopes) the one which uses the maximum percentage of waste effluent is found out to consist of 53% of WE and 47 % of mineral salt medium. This particular composition of WE: mineral salt medium is upgraded to the 1.5 litre bioreactor scale for ultra pure P(3-HB) production. Figure (4) shows the purity of the polymer obtained by the chloroform – sodium hypochlorite treatment of the cell mass (13 g/l) from the bioreactor, by comparing the crotonic acid produced from it with that produced from P(3-HB) manufactured by Aldrich chemical and P(3-HB) procured under the trade name of Biopol from Metabolix, Mirel. Since the fermentation in this process continues only for 2 days (48hours) the energy consumption drops substantially, compared to other conventional methods which continue the fermentation for over 5 days [18]. Secondly from the data presented in Table 2, we can see that there is a substantial reduction in the COD and the BOD of the waste effluent after the fermentation is over. In fact the liquid waste obtained after the fermentation could be recycled in the fermentor itself to minimize the waste disposal problem.

V. Conclusion

As the data presented corroborates, it would not be wrong to be optimistic about producing commercially viable amounts of P(3-HB) from the spent flow of an industry in near future . In fact it may be quite feasible to set up a pilot plant to generate P(3-HB) from sugar industry waste effluent in any sugar industry which uses extraction techniques comparable to this one. As far as the authors know cheap carbon substrates like molasses, curd whey has been previously used as feed stock for P(3-HB) production but never the spent run of any agro industry has been harnessed for this purpose [19].

Acknowledgement:

The authors would like to thank Mr. Anand Dodabasannavar, Quality Control Manager of Shree Renuka Sugars for kindly providing the waste effluent and technical know-how of the sugar plant. Ms. Sarmistha Mukherjee of Kolkata Corporation is also thanked for her help in procurement of the scum. The project has been financially supported by the Green Scheme of DST, Sanction No. SR/S5/GC-12/2009. DBT-IPLS is thanked for providing fellowship to Ms Keka Sarkar.

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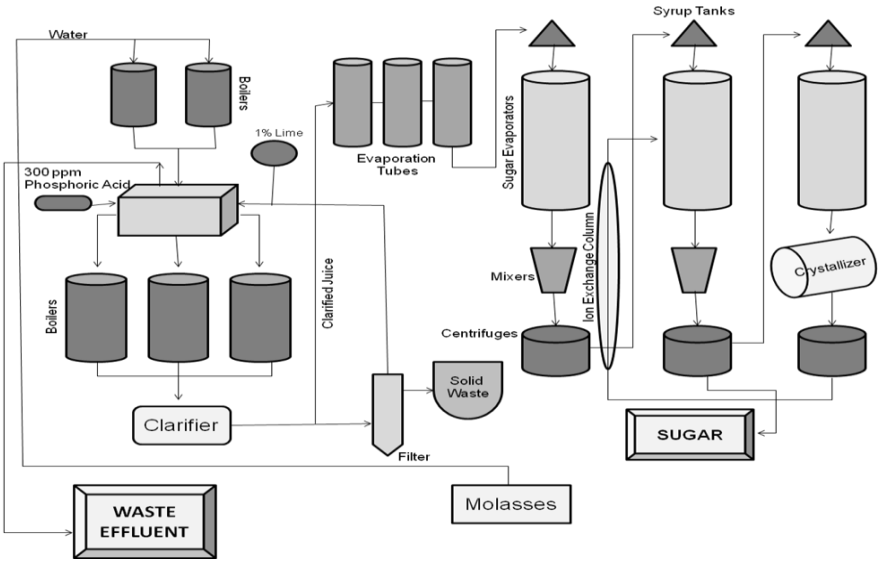
Table 1 Chemical analysis of the waste effluent (WE)

Constituent	Method	Concentration
Glucose content (w/v)	Estimated Using Fehling's Solution	**2.047** ± 0.074 %
	Estimated Using Anthrone Reagent	**2.146** ± 0.004%
Sucrose content (w/v)	Estimated Using Fehling's Solution	**1.089** ± 0.111 %
	Estimated Using Anthrone Reagent	**1.13** ± 0.07 %
Calcium ion (w/v)	Complexometric Titration	**0.021** ± 0.00015%
Vitamin C (Ascorbic acid) (w/v)	Iodometric Titration	**0.047** ± 0.0033 %
Phosphate ion	Spectrophotometric Method	**26** ± 4 ppm

Table 2 Parameters measured that show changes in chemical nature of waste effluent (WE) due to fermentation.

Parameters	Before Fermentation	After Fermentation
COD	50416 ppm	12600 ppm
BOD	28951 ppm	6119 ppm
Total Carbon Content	9720 ppm	4779 ppm



(a)

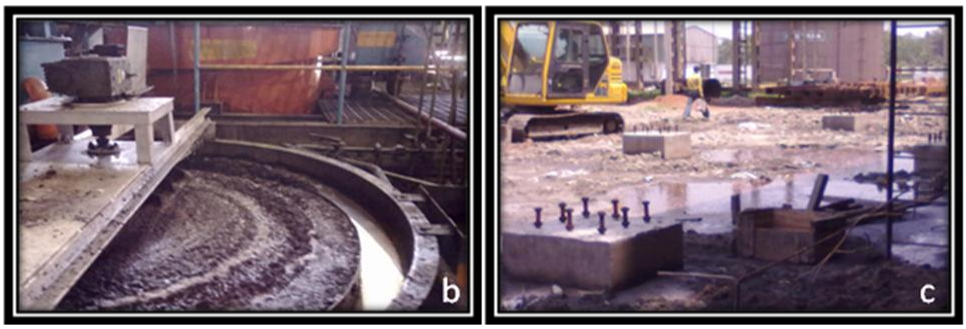


Figure 1. (a) Schematic diagram of sugar production in Shree Renuka Sugar mills. (b) Molasses Clarifier system in the industry. (c) Waste effluent cess pool.

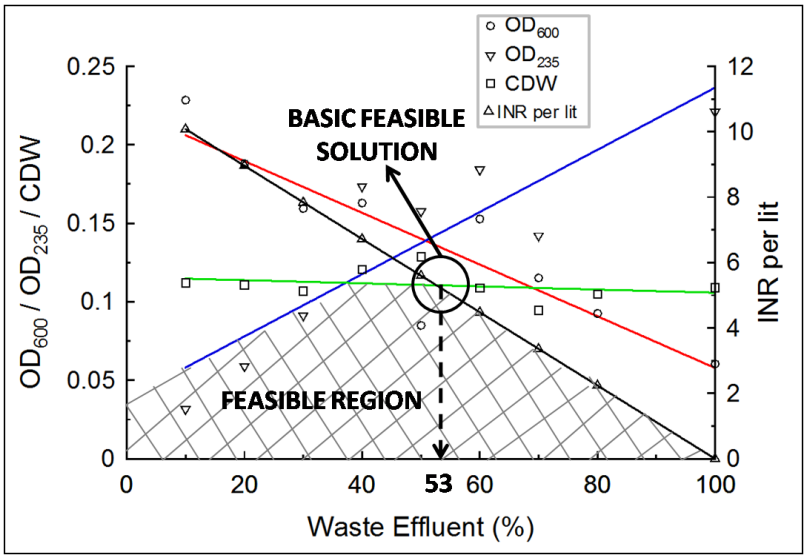


Figure 2. Determination of basic feasible solutions (o) amount of cell accumulation measurement O.D. at 600 nm from 1 ml of 48 hours growing BP/SU1 culture in different concentration of WE, (▽) quantitative estimation of P(3-HB) from 4 ml of culture by oxidizing it to crotonic acid and measuring O.D. at 235 nm from different concentration of WE, (□) amount of cell dry weight [CDW(gm)] generated in different concentration of 5 ml of WE/ mineral media under the same growing condition (△) cost factor in rupees as a function of different concentration of WE in the feedstock for P(3-HB) production

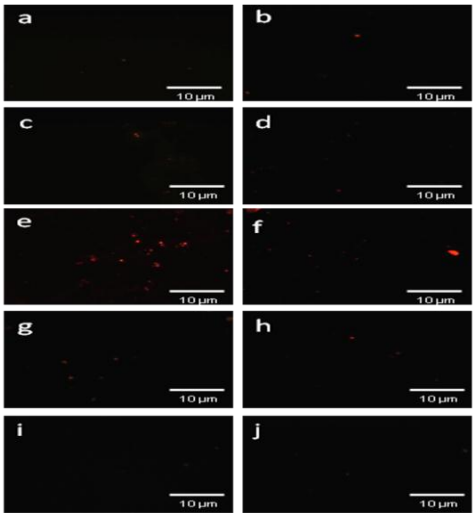


Figure 3. Photomicrographs of BP/SU1 containing P(3-HB) inclusion bodies by Nile blue A staining of 48 hours old culture comprising of (a) 10% WE, (b) 20% WE, (c) 30% WE, (d) 40% WE, (e) 50% WE, (f) 60% WE, (g) 70% WE, (h) 80% WE, (i) 90% WE, (j) 100% WE

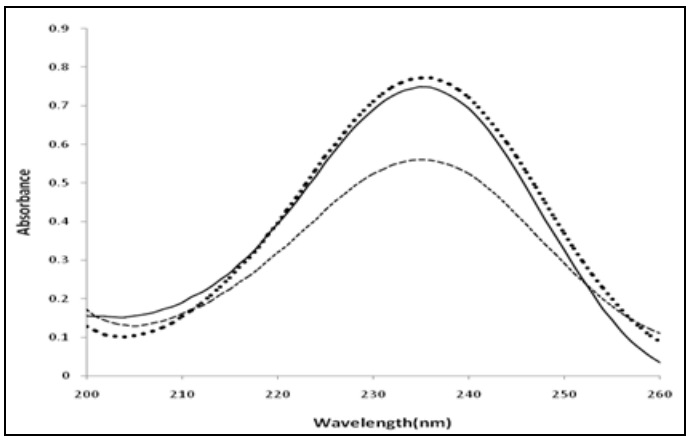


Figure 4. Spectrophotometric observation of crotonic acid peak at 235 nm, P(3-HB) from Aldrich (——); Metabolix MBX D411G (------); P(3-HB) extracted from BP/SU1 cultivated in 53% WE mineral media . (– –)