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SUGAR MILL INDUSTRY EFFLUENT AS A SUBSTRATE FOR POLYHYDROXYALKANOATE (PHA) PRODUCTION

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are natural biodegradable polyhydroxyesters mainly produced by a large array of bacterial species under nutritional stress or limiting conditions. PHA has salient properties like biocompatibility, non-toxic nature, high tensile strength, biodegradability, good ultraviolet resistance and water insolubility. Sugar industry is the backbone of the rural industrial economy, the waste generated in the production process contains severe pollutants. The current research focuses on utilization of this sugar mill effluent as a substrate for the production of PHA from bacteria. The study also demonstrates the standardization of various parameters which help in utilization of sugar mill effluent for maximum PHA production. Maximum PHA yield was obtained at pH 7.5 with peptone as a nitrogen source, when supplemented with 40% SME. Lab scale batch fermentation yielded 35.2% PHA from dry cell weight. Thus, the novel utilization of sugar mill effluent as a substrate for PHA production is demonstrated. This allows potential cost reduction in the polymer production process while also remediating the industrial effluent.

KEY WORDS : Polyhydroxyalkanoates, Biodegradable, Sugar mill effluent, Sustainable development

INTRODUCTION

Treatment of sugar mill effluent is carried out by physicochemical methods like chemical oxidation, coagulation, flocculation, ultrafiltration, reverse osmosis, electrooxidation and electrocoagulation (Singh *et al.*, 2019; Sahu, 2017). These processes are expensive, require a lot of energy and harsh chemicals. The effluents consist of sugars and volatile fatty acids. These components can be remediated by biological treatment methods (Kushwaha, 2015). Due to these reasons, sugar mill effluent has been utilized as a substrate for biogas production (Kumar *et al*., 2020).

Polyhydroxyalkanoates (PHAs) are natural biodegradable polyhydroxyesters which are produced by a large array of microorganisms as lipid inclusions as an energy source in nutrient limiting conditions (Poli *et al.*, 2011). This polymer accumulates in various naturally occurring bacteria. Due to its unique properties like biodegradability, good ultraviolet resistance, high tensile strength, non-toxicity, piezoelectric and water insolubility, extensive studies have been conducted on PHA (Bugnicourt *et al*., 2014) (Raza *et al*., 2018). These properties make it a suitable replacement of traditional petroleum based polymer (Zhuang *et al.*, 2014). A biopolymer like PHA having such salient features is still not commercially viable because of its high production cost, due to expensive raw materials. Mass application of PHA can be achieved by lowering the cost of production.

The study is aimed at isolation of bacterial strains from sugar mill effluent and the further utilization of the waste as a novel substrate for production of PHA. This will ultimately lower the cost of raw materials and potentially remediating the effluent itself. The study also determines the optimum conditions for maximum PHA production when using SME as a substrate.

MATERIALS AND METHODS

Sample collection and isolation microorganisms

The sugar mill industrial effluent was acquired from a sugar mill situated on the outskirts of Karnal district in Haryana (29.6857° N, 76.9905° E) in plastic bottles which were previously sterilized. The pH and the TDS of the effluent was checked and effluent was refrigerated for further processing. For the isolation of putative PHA producing bacterial strain, 1 ml of the effluent was mixed in 9 mL of double distilled autoclaved water and was subjected to 10-fold serial dilution. The strains were isolated by spreading 10^{-6}, 10^{-7} and 10^{-8} dilution on nutrient agar plate (García-Armesto *et al.*, 1993). The plates were incubated for 24 – 48 h. Post incubation each morphologically distinguishable colony was selected and was further streaked on a different NA plate for the isolation of pure bacterial cultures.

Screening of bacterial strains for PHA production

The isolates were individually screened for their PHA production potential by using Nile blue A dye. Each isolate was spotted on agar plates containing Nile blue A (Spiekermann *et al.*, 1999). Incubation of the plates was carried out at 37 °C for 72–96 h. Following this, the plates containing the bacterial cultures were visualised under UV light. The colonies emitting fluorescence were putative PHA accumulators and hence, a clear and strong distinction between PHA-positive and PHA-negative bacterial strains was established.

Selection of bacterial isolates capable of utilizing SME for PHA production

SME - based PHA induction medium was prepared containing SME as a carbon source, $MgSO_4$ (0.2g/l), Peptone (1g/l), $CaCl_2$ (0.2g/l), KH_2PO_4 (1g/l). The pH was set at 7.0±0.5. Inoculum of each isolate was prepared in nutrient broth and introduced to SME-PIM at a concentration 2% (v/v). Incubation of the flasks was carried out at 37 °C for 72-96 h. The bacterial cells were pelleted for the extraction of PHA. The pelleted cells were treated with sodium hypochlorite (Heinrich *et al.*, 2012) and boiling chloroform (Kunasundari and Sudesh, 2011). The chloroform was allowed to evaporate to obtain PHA (Ramsay *et al.*, 1994).

Maximizing PHA production by optimizing cultural and physical conditions

The bacterial strain which yielded the maximum PHA was selected for optimization of the cultural and physical conditions to maximize the production of PHA from SME. Various factors like pH of the medium, nitrogen source and percentage of the SME contribute towards maximizing the yield of PHA (Singh Saharan *et al.*, 2014). Optimization of the following conditions was done, substrate concentration (5%-75%), initial pH (3.0-10.0), incubation time (24–96 h), shaking vs stationary. Different organic and inorganic nitrogen sources were tested, namely Ammonium Chloride (NH_4Cl) and Ammonium Sulphate ($(NH_4)_2SO_4$) whereas the organic sources of nitrogen included Urea, Peptone, Tryptone, Beef extract and Yeast extract. Dry cell weight (mg/l) and PHA dry weight (mg/l) were recorded for each parameter.

Gas chromatography-Mass spectrometry (GC-MS) analysis

GC-MS analysis of PHA is done for the qualitative studies of PHA in bacterial cells. Sample for GC-MS analysis was prepared by carrying out methanolysis of PHA. This was achieved by suspending the biopolymer in equal parts chloroform and methanol containing 2.8 M of sulphuric acid (H_2SO_4) in a glass screw-capped tube (Huang *et al.*, 2018). This mixture was incubated at 100 °C for 2 h. Post incubation, double distilled water was added and the mixture was vortexed for 20 – 30 s in order to mix the contents uniformly and was then centrifuged to facilitate phase separation (Lee and Choi, 1997). Commercially available polyhydroxyalkanoic acid (Sigma) was used as a standard and the sample for analysis was prepared in a similar manner as the biopolymer extracted from the bacterial cells. The organic phase containing methyl esters of biopolymeric monomers from both standard and the sample was collected and was analysed by GC-MS. The organic phase was transferred into GC vials for analysis using GCMS-QP2010ULTRA with RTX-5 column and FID detector. Electron ionization system was operated in an electron impact form for GCMS detection with an ionization energy of 70eV. Mass spectrum was analysed using National Institute Standard and Technology (NIST).

RESULTS AND DISCUSSION

Industrial effluent, isolation and screening of bacterial isolates

Collection of sugar mill industrial effluent (SME) was done in sterile plastic bottles. The effluent was

opaque dark brown in colour with decaying fruity smell, a pH of 5.2 and TDS was found to be 4050 ppm. The industrial effluent collected from the sugar mill was the waste generated in course of clarification of syrup concentration and cooking of the sugar cane juices during sugar manufacturing process. Sixteen distinct strains were isolated from the industrial effluent, 4 bacterial strains emitted fluorescence and thus were selected as putative PHA producers.

Utilization of SME and optimization of conditions for maximum PHA production

Two PHA producing isolates were able to utilize SME as a substrate. PHA films of differing weights were obtained. Thus, these isolates were able to utilize SME as a source of carbon to synthesize PHA. Out of the two strains, the bacterial strain named ART_36N was the highest PHA producing isolate as it yielded more PHA than the other bacterial strain.

ART_36N bacterial isolate was selected for optimization of the cultural and physical conditions to maximize PHA production. Each process parameter was varied and the condition at which maximum PHA was obtained, was chosen for further investigations. The maximum PHA extracted was 116 mg/l at a pH of 7.5 (Fig. 1). Maximum PHA yield of 124 mg/l was obtained from media containing peptone as the nitrogen source, while no PHA was obtained when the isolate was grown in medium containing inorganic sources of nitrogen (Fig. 2a, 2b). Substrate concentration was optimized at 40% (v/v) SME which led to 152 mg/l PHA accumulation (Fig. 3a, 3b). Incubation times was optimized at 72 h because it resulted in maximum PHA production of 112 mg/l (Fig. 4). Similarly, maximum PHA extraction occurred in agitated condition which resulted a yield of 96 mg/l (Fig. 5). Thus, these conditions were incorporated to confirm the ability of ART_36N to produce 35.2% PHA of its cell dry weight (Table 1).

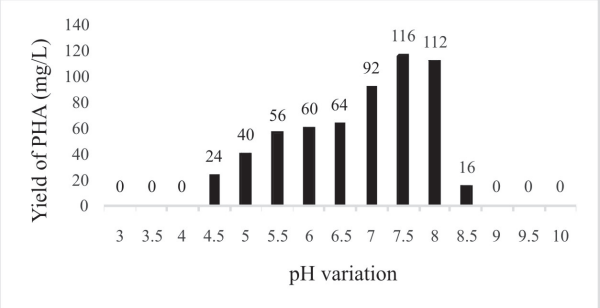

Fig. 1. Effect of pH variation on yield of PHA

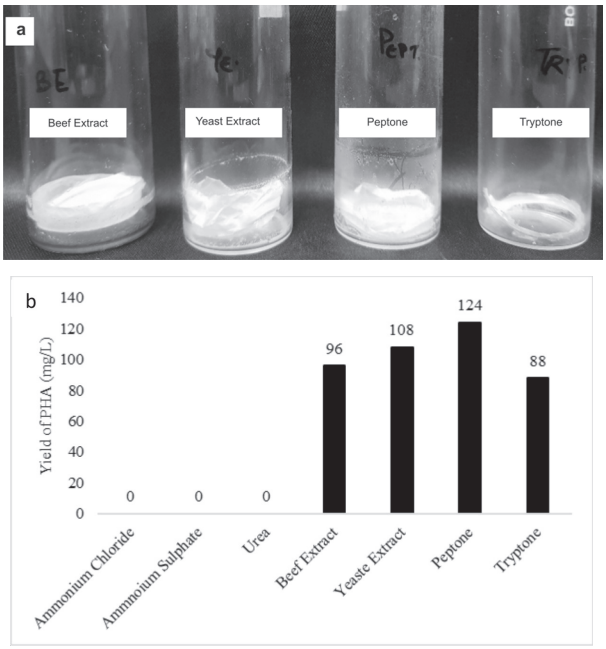

Fig. 2. a) Effect of nitrogen source on PHA production b) Effect of various nitrogen sources on the yield of PHA

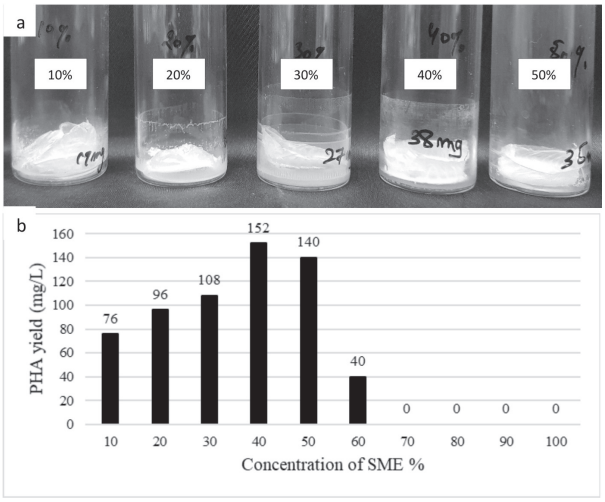

Fig. 3. a) Effect of SME concentration on PHA production b) Effect of concentration of SME on PHA yield

Table 1. Determination of cell dry weight and PHA yield percentage

Cell dry weight (g/l)	PHA produced (g/l)	PHA yield (%)
1.08	0.38	35.2

Gas chromatography-Mass spectrometry (GC-MS) analysis

GC-MS analysis allowed us to observe that the commercial standard and the biopolymer extracted

from the bacterial cells have the same methyl esters of the polymeric monomers. The peaks were obtained at the same time interval and had the same retention time. The commercial standard (3-hydroxybutyric acid) had characteristic peaks of hexadecenoic acid and octadecanoic acid at 22.186, and 27.239 respectively. The extracted biopolymer had similar retention peaks of hexadecenoic acid and octadecenoic acid at 22.220 and 27.295, respectively. Since the polymer extracted from the bacterial cells had the same methyl esters of polymeric monomers with peaks occurring at same interval and having same retention time as the commercial standard, it can be concluded that the bacterial polymer was PHB (Hiroe *et al*., 2016) (Table 2 and Fig. 6).

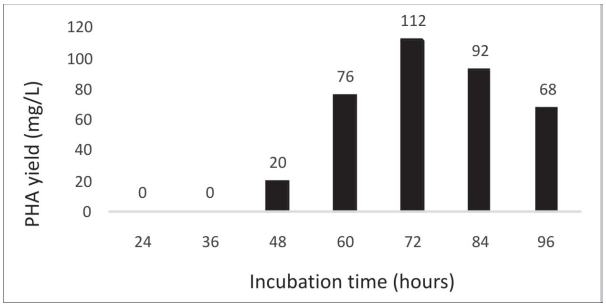

Fig. 4. Effect of incubation time on PHA yield

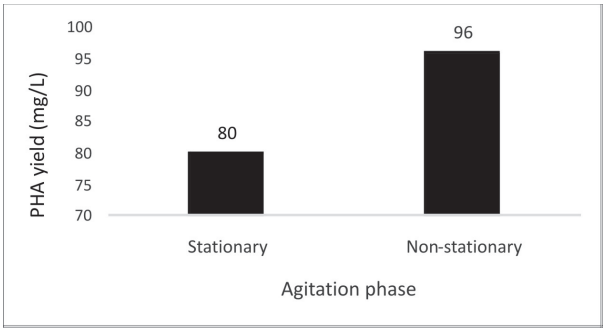

Fig. 5. Effect of agitation phase on PHA production

CONCLUSION

This study exhibits the isolation of bacterial strain from sugar mill industrial effluent. The isolation of bacterial strains was done in order to obtain a

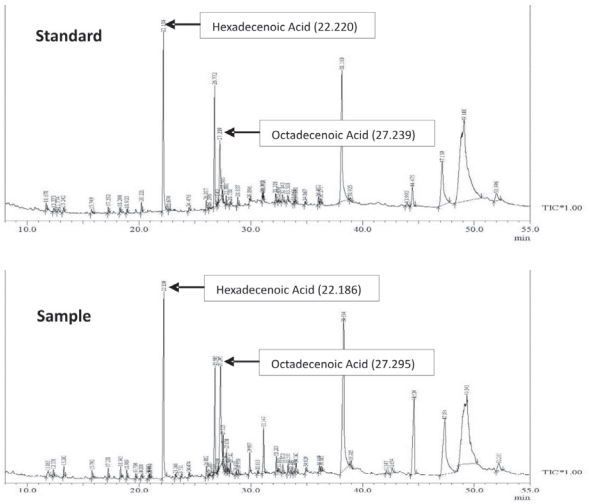

Fig. 6. Comparative GC-MS analysis of standard and extracted biopolymer

bacterial strain capable of utilizing SME as a substrate for PHA production. Sixteen bacterial strains were isolated, ART_36N was found to be the highest PHA producing isolate. Cultural and physical parameters were optimized which showed that ART_36N produced maximum PHA at a pH of 7.5 with peptone as a nitrogen source, when supplemented with 40% SME and incubated for 72 h under shaking conditions. The resultant yield was 35.2% PHA of cell dry weight. Qualitative analysis was done with the help of GC-MS which conclusively shows characteristic peak of hexadecenoic and octadecenoic acids in the standard and the biopolymer extracted from ART_36N at same interval and same retention time. Thus, we demonstrate SME can be utilized as a novel substrate for PHA production using the isolate ART_36N isolated from the waste itself.

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Table 2. Comparative analysis of retention time and area of standard and polymer

Methyl Esters	Retention Time (min)		Retention Area (%)	
	Standard	Extracted Biopolymer	Standard	Extracted Biopolymer
Hexadecenoic Acid	22.186	22.220	10.55	9.67
Octadecenoic Acid	27.239	27.295	5.65	5.52

The authors have no conflict of interest to declare.

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