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BIOPROCESSING BRILLIANCE: UNVEILING PSEUDOMONAS AERUGINOSA'S ROLE IN OPTIMIZING PHA FROM WASTE FRYING OIL

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Abstract
The increasing amount of plastic waste and its negative impact on the environment has led to the development of biodegradable plastics, such as polyhydroxyalkanoates (PHA). PHA is a biodegradable polyester synthesized by bacteria and has garnered attention as a solution to the plastic waste problem. In this study, we aimed to optimize PHA production from waste frying oil using Pseudomonas aeruginosa. We compared the PHA production of P. aeruginosa and Bacillus subtilis, the two screened microorganisms, using different carbon sources including rice bran, paddy husk, pigeon pea waste, sugarcane bagasse, and waste frying oil. Our results showed that P. aeruginosa produced more PHA than B. subtilis, and waste frying oil was the most effective carbon source. Moreover, increasing the concentration of hydrolyzed carbon source led to higher PHA production. We confirmed our findings through nitrogen estimation, microscopic observation, and gas chromatography analysis. Our study suggests that PHA can be produced from waste frying oil by P. aeruginosa with high efficiency, proving promising for its potential use as an eco-friendly alternative to traditional plastics.

Keywords: PHA, biodegradable plastics, waste frying oil, Pseudomonas aeruginosa, carbon source.

I.INTRODUCTION

The accumulation of plastic wastes has become a major concern in terms of the environment(1). Since synthetic plastics marked their debut in the 1950s, they have emerged to be among the most needed material in our daily life(2). The extensive usage of petrochemical plastics due to their versatile properties especially durability is causing severe problem in waste management affecting the aesthetic quality of cities, water bodies and natural areas(3). Problems concerning the global environment have created much attention in developing eco-friendly products .Biopolymers are one product that can help to overcome problems caused by petrochemical polymers. Biopolymers are generated from renewable natural sources and are often biodegradable and nontoxic(4).

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters synthesized by various bacteria They represent products with biotechnological importance due to their special properties .They are accumulated intracellular as carbon and energy reserves under certain conditions(5).

PHA being biodegradable and biocompatible has application that varies from industries to medical therapeutics. Microorganisms accumulate PHA as an intracellular reserved material in response to imbalance in the growth environment where a suitable carbon source is present in excess and one or more nutrients are limiting , example, nitrogen, phosphorus , sodium, oxygen, magnesium, manganese.(6)

PHA production from renewable carbon sources either by microbes or plants has become a valuable commercial prospect due to lower carbon feedstock costs, since PHAs were first identified as environment friendly biodegradable plastics

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II. Materials and Methods

2.1 Collection of organism and subculturing

The organisms *Pseudomonas aeruginosa* and *Bacillus subtilis* were collected from Biogenics research centre, Hubli. The subculturing of organisms were done in 25 ml of nutrient broth. The nutrient broth contained 0.25gm peptone, 0.25gm NaCl and 0.125gm yeast extract in 25ml distilled water.The media was sterilized at 120˚c and 15 lb pressure. The media was cooled to room temperature and inoculated with the bacterial samples under laminar air flow. Two sets of media were prepared for these two samples and were incubated at room temperature on rotary flask shaker at 150 rpm for 72hours. The graph is shown in figure 1.

2.2. Screening of microorganisms

2.2.1. Mineral media preparation

The mineral media was prepared with the following composition: 0.01gm yeast extract, 0.1gm substrate(dextrose), 0.64gm $NaHPO_4.7H_2O$, 0.15gm K_2HPO_4, 0.025gm NaCl, 0.05gm NH_4Cl, pH 7, 1ml of 1M $MgSO_4$ stock solution, 1ml of 0.1M $CaCl_2$ stock solution in 50 ml distilled water(7). Media was sterilized and inoculated with each bacterial sample in separate flasks and were incubated on rotary flask shaker at 150 rpm for 72hours at room temperature.
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2.2.2. Microscopic observation

Microscopic analysis of heat- fixed cells was done after staining with crystal violet for 10sec and destaining with running water for 10sec(8).The clumps were observed under compound microscope, as shown in figure 2.

2.2.3. N_2 estimation and extraction

The samples were centrifuged at 10,000 rpm for 10 minutes. The cell pellets were taken for extraction and the supernatant was taken for N_2 estimation.
For N_2 estimation Nesslers reagent was used and OD was measured at 500 nm. (9)The graph was plotted by using standard nitrogen values as shown in figure 3.
For extraction the cell pellets were washed with distilled water and dried at 90˚C in incubator for 3hrs. Later, cell pellets were collected from tubes by scrapping and weighed. Further, 0.6ml of(9ml methanol+0.3ml H_2SO_4+1ml) mixture was added into the vials containing 0.01gms of dried cell pellets and heated at 90˚c for 2 hours. The mixture was then cooled to room temperature. Then 0.6 ml of chloroform and 0.3 ml of distilled water was added and mixed slowly for cell separation. Centrifugation was carried out at 8000 rpm for 1min, the top chloroform layer containing PHA was collected into a different vial and was taken for analysis using GC-MS shown in figure4.(10)
Gas chro matography mass spectral (GC-MS) was performed with SHIMADZU QP2010S (KUD, Dharwad).The mass spectrometer was auto tuned with decafluorotriphenylphosphine and a70 x 106 eV fragmentation energy. Spectra were recorded at a scan speed of 380atomic mass units (AMU)/s (4 samples per 0.1 AMU) with a 0.5-s delay between scans of 50 to 500 AMU. The spectrometer was operated in the peak finder mode at an electron multiplier voltage of 1,800 V. The threshold of detectability was 200 linear counts.

2.3. Screening of substrate

The easily available cheap carbon sources like rice husk, paddy waste, sugarcane baggasse, pigeon pea waste and waste frying oil were collected from the villages of Karnataka (India). The substrates were grinded into fine powder (except oil). Hydrolyzation of these substrates were done by adding 50ml of mixture containing 750 µl of H_2SO_4 in 100 ml distilled water to each 50gms of powdered substrates and diluted to 1:4 ratio. The flasks containing hydrolyzed carbon were autoclaved and these samples were squeezed using muslin cloth and the filtrate was taken for centrifugation (10,000rpm for 10 min). The supernatant was used as carbon source in mineral media for the

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production of PHA in combination with *Pseudomonas aeruginosa*. Further procedure for production and extraction of PHA was same as followed in the screening of microorganisms. Analysis was done using GC-MS, the graphs are as shown in figure 5.

2.4. Effect of carbon source

Carbon source plays very important role in increasing the concentration of PHA, hence a study of effect of carbon source was performed by increasing the concentration of hydrolyzed carbon source 5 times and 10 times of that used above for the screening of carbon source. GC- MS was performed for both the samples. Graph is shown in figure 6.

III. RESULTS AND DISCCUSSION

3.1. Growth rate studies of organism:

The growth behavior of individual organism was observed by preparing the nutrient broth, inoculation, incubation and determination of optical density at different time intervals.

The growth pattern exhibited by the individual organism is depicted in the following graph which clearly marks higher growth rate of Pseudomonas aeruginosa over Bacillus subtilis.

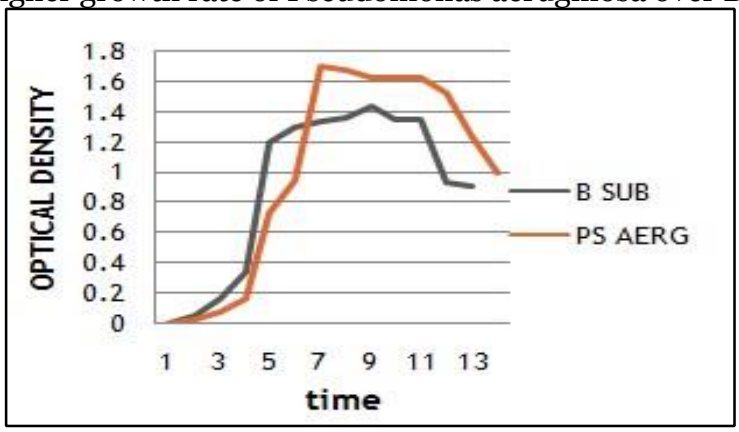

Fig. 1 Growth rate studies of organisms

3.2. Nitrogen estimation:

It has been reported that the nitrogen concentration in bacteriological media highly influences the production of intracellular PHAs (Khanna and Srivastava 2005b). From the experimental analysis and comparison of standard nitrogen graph it was found that about 616µg and 328µg of nitrogen was present in the organisms *Pseudomonas aeruginosa* and *Bacillus subtilis*.

Table1: Estimation of nitrogen

Sl No	Sample(ml)	H2O(ml)	Neslers reagent (ml)		OD(500nm)
1	00	2.7	0.3	INCUBATION	00
2	0.2	2.5	0.3	AT ROOM TEMP	0.317
3	0.2	2.5	0.3	FOR 20 MIN	0.592

Table2: Standard nitrogen table

Conc. of ammonia(µg)	OD at 500 nm
00	00
20	0.16

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40	0.31
60	0.45
80	0.59
100	1.22

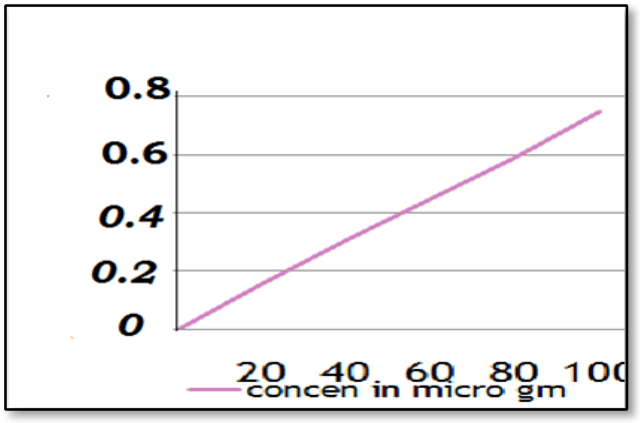


fig: Standard nitrogen curve

3.3. (a)Microscopic observation:

Incubation of bacterial samples for 72 hours and staining it with crystal violet revealed the dark clumps of the violet colored cells under microscopic examination , thus qualitatively confirming the production of PHA polymer by the organisms(7).

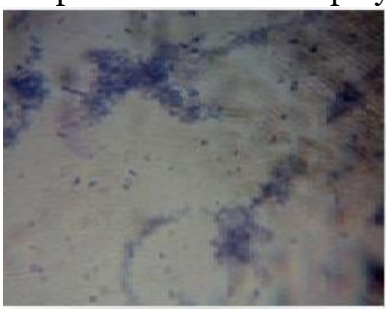


Fig: clumps of *Pseudomonas aeruginosa*

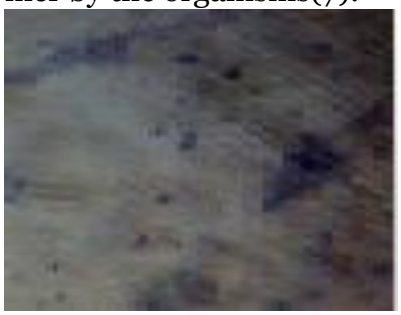
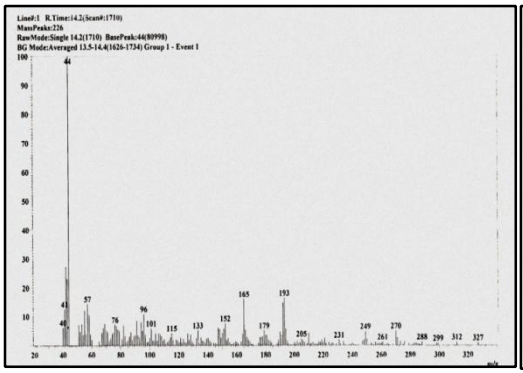


Fig: clumps of *Bacillus subtilis*

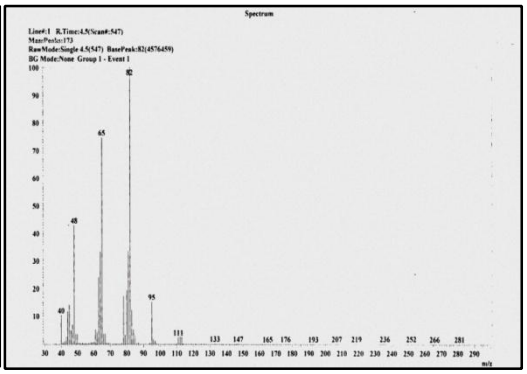
(b)Gas chromatography analysis:

Screening of microorganisms

Bacillus subtilis



Pseudomonas aeruginosa



Screening of substrates

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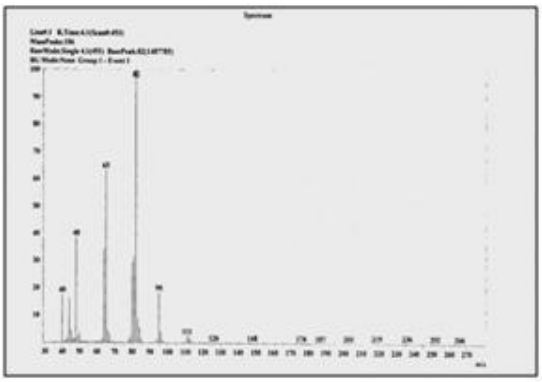
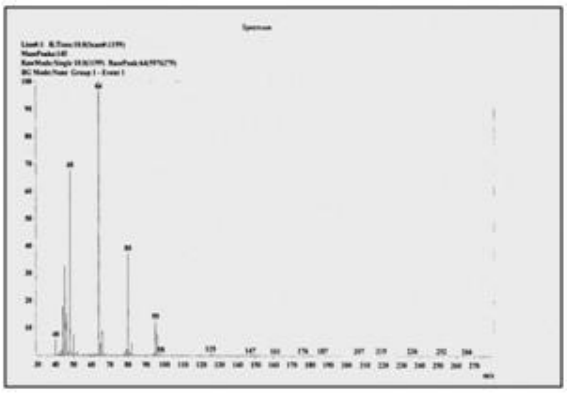
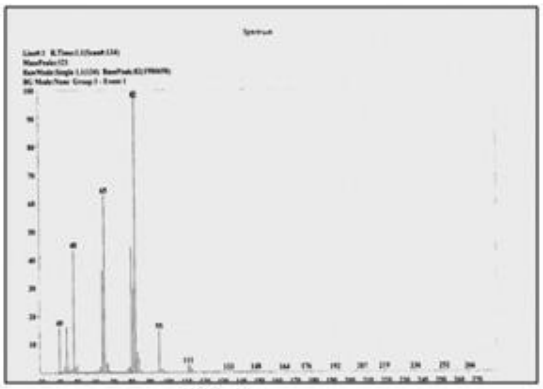
Screening of substrates



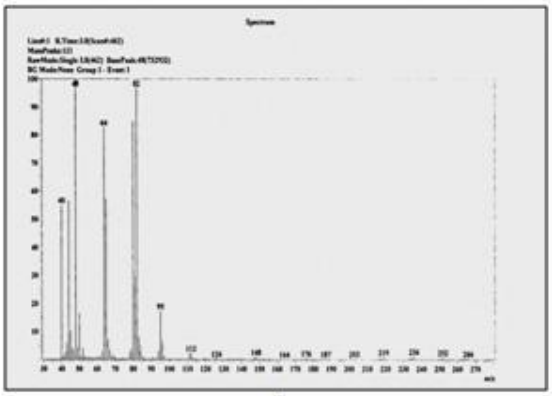
Paddy waste



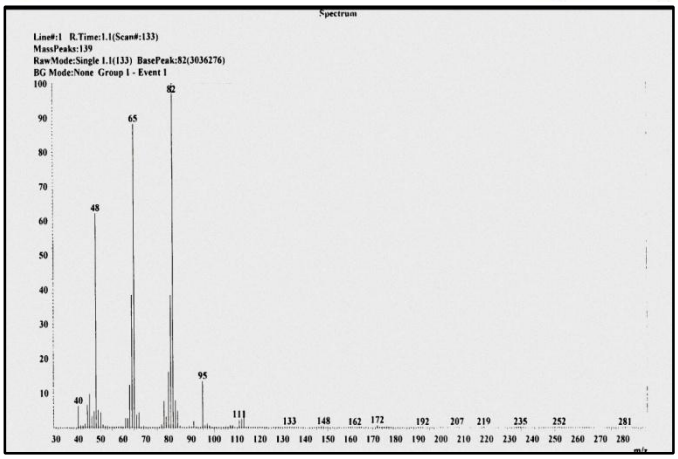
pigeon pea waste



Rice bran



sugarcase bagasse



Waste frying oil

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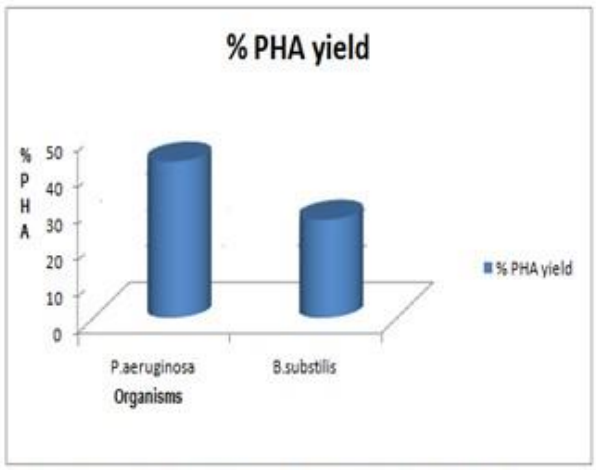
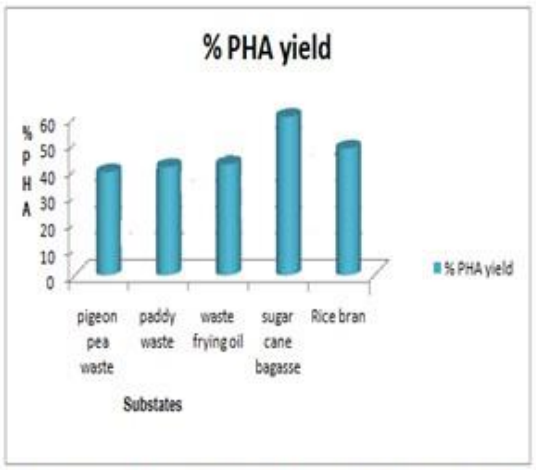


Percentage PHA yield by *P.aeruginosa* and *B.subtilis*



Percentage PHA yield by different Substrates with *P.aeruginosa*

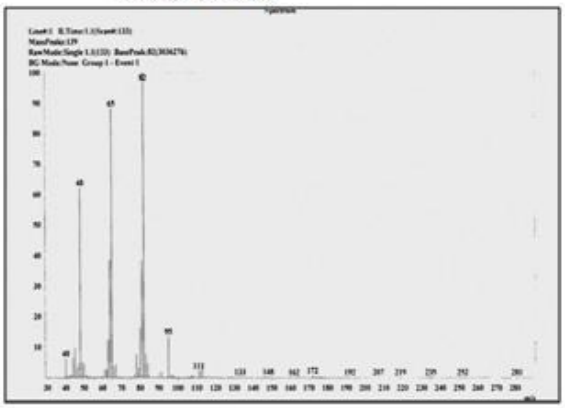
From the GC-MS graphs the height of the peaks indicates the concentration of PHA present in the different organisms. According to the literature survey the molecular weight of PHA should not exceed 120 m/z. The graph of Bacillus subtilis showed many different peaks and the peaks exceeded 120m/z. Hence it indicates the presence of other lipid contaminants. Where as in the graph of Pseudomonas aeruginosa the peaks are clear and are not exceeding 120m/z. The result in the form of the bar graph clearly shows the presence of the percentage of PHA in both the organisms.

The percentage of PHA in Pseudomonas aeruginosa was found to be 50% and the same in Bacillus subtilis is 30%. Hence we conclude that among the two, Pseudomonas aeruginosa can produce more PHA and was screened as the best organism for the production of polyhydroxyalkanoate.

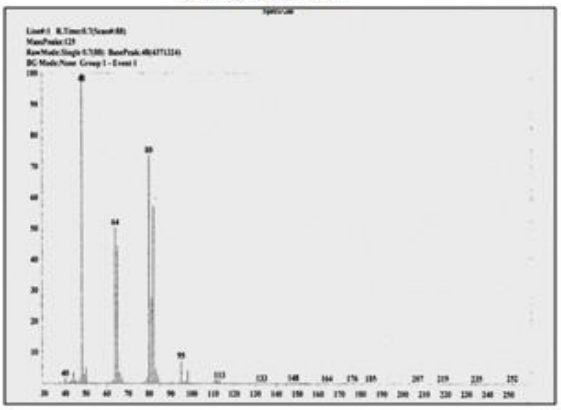
From the graph *Pseudomonas aeruginosa* with the combination of five different substrates i.e pigeon pea waste, paddy waste, waste frying oil, sugarcane baggasse and rice bran produced about 39%, 41%, 42%, 60% and 48% of PHA.

There by we conclude that *Pseudomonas aeruginosa* in combination with Sugarcane baggasse produce the maximum concentration of PHA. Hence sugarcane baggasse was screened as the best substrate for the production of PHA with *Pseudomonas aeruginosa.*

5 times increase



10 times increase



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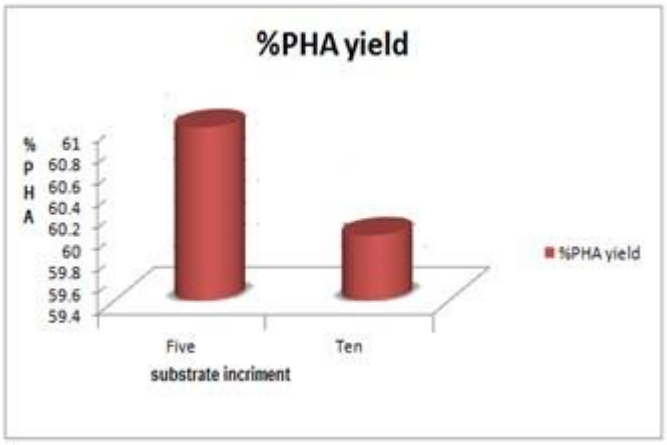

Percentage PHA yield by increase in substrate with combination of sugarcane bagasse
With P.aeruginosa

Carbon source plays a very important role in the production of PHA. Increase in the concentration of carbon source by five times produced 61% of PHA and the production was reduced to 60% when the concentration was increased ten times. This is due to the attainment of saturation level, more concentration of carbon source lead to the death of micro organism.

Pseudomonas aeruginosa accumulated the maximum yield of 60% PHA when sugarcane bagasse was used as carbon source. This result is very similar to the report of Ogunjobi, A.A.et al, (2011) who reported the yield of 55.68% by *Pseudomonas putrefaciens* with sugarcane baggasse. This result is also very similar to the report of Suzuki *et al.,* (1986) who reported maximum yield of 66% w/w dry weight by a *Pseudomonas* sp. when methanol was used as sole carbon substrate for PHA production. The use of methanol as a carbon source is significant because it is such a cheap carbon source and could reduce production cost. However, sugarcane bagasse allowed the highest biomass density in this bacterial strain and the PHA yield obtained was equally high. This observation further supports the reports of Santhanam and Screenivasan, (2010) that a higher concentration of PHA polymer obtained with commercial sugar suggests the possibility of using cane molasses or other cheaper sucrose substrates in future.

IV. Conclusion

PHA content and its composition are influenced mainly by the strain of microorganism, the type of substrate employed and its concentration, and environmental growth conditions. The production of biodegradable polyesters on a large scale is limited because of the relative expensive substrate required, low polymer production per unit of substrate utilized, and the cost of maintaining pure cultures. Higher production costs, especially raw material costs, make it difficult for PHA biodegradable plastics to compete with conventional petroleum-base plastics in the commercial marketplace. Sugarcane bagasse, a readily available bio-waste material was found to be the most suitable carbon substrate on selected bacterial species used in this study, as it enhanced the highest polymer yields. Hence, the production of these environmentally-friendly materials can compete with synthetic plastics on a large-scale if the cost of fermenting substrate, which contributes significantly to the overall production cost, has been reduced considerably.

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