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Production of polyhydroxyalkanoates by *Ralstonia eutropha* from volatile fatty acids

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Abstract−Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible thermoplastics that can be synthesized in various microorganisms. Volatile fatty acids (VFAs) are produced by anaerobic treatment of organic wastes that can be utilized as inexpensive substrates for PHA synthesis. In this study, several *Ralstonia eutropha* strains were grown on the mixture of VFAs (acetic, propionic, and butyric acid) as its carbon and energy source for growth and PHA synthesis. *R. eutropha* KCTC 2658 accumulated PHAs up to 50% of dry cell weight from total 5 g/L of mixed VFAs (acetic acid : propionic acid : butyric acid=1 : 2 : 2). In batch culture of *R. eutropha* KCTC2658 in a 5 L fermentor, a homopolymer of poly(3-hydroxybutyrate) [P(3HB)] was produced from 20 g/L glucose as a sole carbon source with dry cell weight of 8.4 g/L and PHA content of 30%. In fed-batch culture, two feeding strategies, pulse or pH-stat, were applied to add VFAs to the fermentor. When VFAs were fed using pH-stat feeding strategy after 40 h, a copolymer of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] was produced with dry cell weight of 8.1 g/L, PHA content of 50%, and 3HV fraction of 20 mol%.

Key words: Polyhydroxyalkanoates, PHA, *Ralstonia eutropha*, Volatile Fatty Acids

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible thermoplastics that can be synthesized in many microorganisms from almost all genera of the microbial kingdom [1]. PHAs usually accumulate within cells in the presence of excess carbon source, and growth is limited by nutrients such as nitrogen, oxygen, and other essential elements. Even though there is considerable interest in PHA due to its potential as a biodegradable material, the wider use of PHA has been restricted by its high production costs [2,3]. Previous authors have reported that the main reasons for high PHA production costs are the need for effective carbon substrates and extraction processes [4]. Almost 30% of the total PHA production cost is attributed to the acquisition of carbon substrates [5].

Research into different types of PHA is beginning to manifest materials with varying potential. The homopolymer poly(3-hydroxybutyrate) [P(3HB)] is known to have high crystallinity, stiffness, and brittleness, and is therefore of limited practical use. However, the inclusion of a fraction of 3-hydroxyvalerate or other monomers in P(3HB) significantly increases the softness and flexibility of this biopolymer [6]. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] copolymer was industrially produced from glucose and propionic acid under the trade name Biopol [7]. The production of this copolymer is generally a high cost process due to the requirement for a specific mixture of carbon sources.

An alternative production method which may reduce PHA production costs is the use of inexpensive carbon sources as feed stocks for the bacteria, such as volatile fatty acids (VFAs). VFAs can be produced from food wastes, sludge, and a variety of biodegradable organic wastes via a VFAs platform [8,9]. Recently, VFAs were used as a sole carbon source for lipid accumulation by *Cryptococcus albidus* [10]. In activated sludge fed by VFAs, the maximum PHA content in the dry cell was 56.5% [11]. PHA composition in mixed culture from fermented molasses was manipulated from 15 to 39% of 3HV by changing VFA composition and feeding regime [12].

Ralstonia eutropha (*Cupriavidus necator*) has been studied most extensively to produce various short-chain-length PHA (SCL-PHA) such as P(3HB), P(3HB-co-3HV), and poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] with various compositions [7,13-15]. SCL-PHA production with high yield and productivity has been achieved in an optimized fermentation by using sugars or vegetable oil as carbon sources [7,13-15]. However, PHA production from inexpensive VFAs has rarely been reported. In this study, several strains of *R. eutropha* were grown to investigate the characteristics of PHA synthesis from VFAs. The optimum strain was selected in terms of final cell concentration and PHA content. Cell growth and PHA accumulation characteristics of the optimum *R. eutropha* strain were investigated in flasks and fermentors of batch and fed-batch cultures in order to improve the production of polyester.

EXPERIMENTAL

1. Microorganisms

The types of strains used in this study were *R. eutropha* (*C. necator*) ATCC 17699 (KCCM 11972, DSM428), KCTC2657 (DSM416), KCTC2658 (DSM418), KCTC2659 (DSM422), KCTC2662 (DSM530), and KCTC2649 (NCIMB11599). All strains were bought from KCTC, activated in LB medium (peptone 10 g/L, yeast extract 5 g/L, and NaCl 5 g/L) and stored with glycerol (15% v/v) in 1.5 mL microtubes at −20 °C.

2. Medium

LB medium was used for seed culture. The mineral medium for the

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flask and batch cultures was composed of $(NH_4)_2SO_4$ 1 g/L, KH_2PO_4 1.5 g/L, $Na_2HPO_4 \cdot 12H_2O$ 9 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, and trace element solution 1 mL/L. The medium for the initial fed-batch culture was composed of $(NH_4)_2HPO_4$ 4.3 g/L, KH_2PO_4 13.3 g/L, citric acid 1.7 g/L, $MgSO_4 \cdot 7H_2O$ 1.2 g/L, and trace element solution 10 mL/L. The trace element solution was composed of H_3BO_3 0.3 g/L, $CoCl_2 \cdot 6H_2O$ 0.2 g/L, $ZnSO_4 \cdot 7H_2O$ 0.1 g/L, $MnSO_4 \cdot 4\text{-}5H_2O$ 0.03 g/L, $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$ 0.03 g/L, $NiCl_2 \cdot 6H_2O$ 0.02 g/L, and $CuSO_4 \cdot 5H_2O$ 0.01 g/L. Mixture of VFAs (acetic acid : propionic acid : butyric acid= 1 : 2 : 2) or glucose was used as carbon sources.

3. Growth Conditions

LB medium was used for pre-cultivation of *R. eutropha*. Cells were grown under aeration for 24 h at 30 °C on a shaking incubator at 250 rpm in 250 mL flasks containing 50 mL of medium. For the flask culture, 1 mL of the preculture was inoculated into 50 mL of mineral medium containing 5 g/L VFAs or 20 g/L glucose as carbon sources. For the batch and fed-batch cultures in the fermentor, 10% v/v of the culture was inoculated into a 5 L fermentor (KoBio Tech, Korea) equipped with a pH meter, a dissolved oxygen (DO) meter, and 1 six-bladed disk turbine impeller. The initial working volume was 2 L. The culture temperature and pH level were 30 °C and 6.5, respectively. The pH was controlled with 5 N NaOH solution. The aeration rate was 2 vvm. DO was kept above 20% by adjusting the agitation speed from 700 to 1,300 rpm. In fed-batch culture, feeding was started after exhaustion of initial glucose supplied (20 g/L) at 36-40 h. Two feeding strategies, pulse or pH-stat feeding, were applied to add VFAs to the fermentor. In the pulse feeding strategy, 5 g/L VFAs was added after exhaustion of initial glucose supplied (20 g/L). In pH-stat feeding strategy, VFAs were automatically added by acid-supplying pump when pH rose above 6.5.

4. Analysis

Microbial growth was monitored by measuring the cell density of the culture at 600 nm after suitable dilution with distilled water. Dry cell weight (DCW) was measured after vacuum drying at −50 °C for 24 h. The amount of PHA content and polymer composition was measured with gas chromatography (GC) according to the following process: About 10 to 20 mg lyophilized biomass was mixed with 2 mL chloroform and 2 mL acidified methanol (methanol : H_2SO_4 : internal standard=850 mL : 150 mL : 2 g). Benzoic acid was used as an internal standard. They were kept in oven at 100 °C for 5 h. After cooling, 1 mL water was added and mixed completely. After layer separation, the bottom phase was used for injection to GC (GC6890N/FID, Agilent) with an HP-5 column (30 m length, 320 μm internal diameter, and 0.25 μm film thickness). The operating conditions of GC were as follows: injection volume=1 mL, initial column temperature=60 °C for 5 min, temperature increase rate=4 °C/min, final column temperature=180 °C for 5 min, carrier gas flow rate=20 mL/min, temperature of injection port=230 °C, and temperature of detection port=280 °C. Glucose and VFAs were analyzed using high-pressure liquid chromatography (HPLC) system (YL 9100, YOUNG-LIN Inc., Korea). Glucose was measured by a Zorbax Carbohydrate Analysis column (Agilent) with a refractive index detector. VFAs were measured by a Zorbax Eclipse Plus C18 column (Agilent) with an ultraviolet (UV) detector at 210 nm.

RESULTS AND DISCUSSION

1. Screening and Selection of Highest PHA Accumulating Strain

Six strains of *R. eutropha* were grown to investigate the characteristics of PHA synthesis from VFAs. Experiments were conducted to select highest PHA producing strain employing VFAs as carbon source. The composition of VFAs used was acetic acid : propionic acid : butyric acid=1 : 2 : 2, one of the ratios of the mixed organic acids produced during the fermentation of food waste. During 60 h incubation from 5 g/L of VFAs, DCW, PHA concentration, PHA content, and 3HV fraction ranged from 0.85 to 1.5 g/L, 0.029 to 0.73 g/L, 4 to 50%, and 0 to 35 mol%, respectively (Table 1). We determined that *R. eutropha* KCTC 2658 was the optimum strain in terms of DCW, PHA concentration, and PHA content. From 5 g/L of VFAs, *R. eutropha* KCTC 2658 produced 0.73 g/L of PHA with highest PHA content of 50% and 3HV fraction of 21 mol%. Even though *R. eutropha* ATCC17699 produced PHA with highest 3HV fraction of 35 mol%, DCW and PHA content were lower (Table 1).

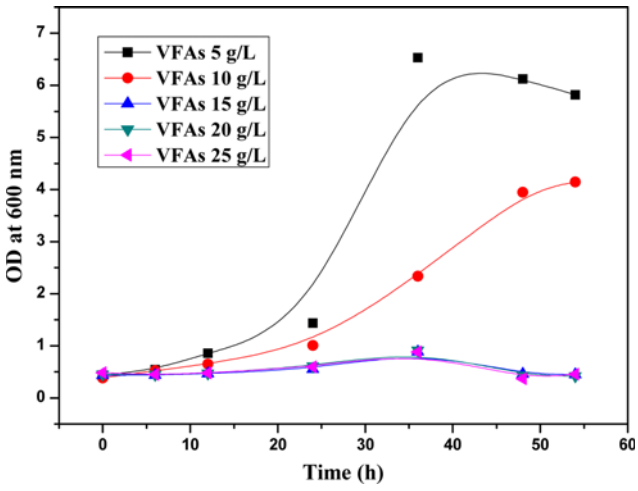

Fig. 1. Growth of *R. eutropha* KCTC 2658 with different initial concentrations of VFAs (5-25 g/L).

Table 1. Screening of *R. eutropha* after 60 h cultivation in flask (initial VFAs concentration=5 g/L)

Microorganism	DCW (g/L)	PHA (g/L)	PHA (wt%)	3HB (mol%)	3HV (mol%)
ATCC17699	1.2	0.30	25	65	35
KCTC2649	0.89	0.029	4.0	100	0
KCTC2657	0.85	0.035	5.0	100	0
KCTC2658	1.5	0.73	50	79	21
KCTC2659	1.2	0.50	42	79	21
KCTC2662	1.3	0.45	36	72	28

2. VFAs Utilization and Inhibition

The VFAs are inhibitory and toxic to the bacterium depending on the total acid concentration [16]. These three VFAs (acetic, propionic, and butyric acids) have almost the same pKa (4.75-4.87) and dissociate to the same extent in the cytoplasm after the undissociated acid molecules penetrate into the cells, which causes the same inhibitory or toxic effect on the cell activity. To check the optimum concentration of VFAs, we conducted an experiment to see their effect on the growth of *R. eutropha* KCTC 2658. Fig. 1 shows the growth curve on different concentrations of VFAs. At 5 g/L of VFAs, growth rate was highest and cells were grown to final OD 6.5. At 10 g/L of VFAs, growth was inhibited with final OD 4. Above 15 g/L of VFAs, almost no growth occurred, suggesting that VFAs should be kept under 5 g/L.

3. Batch Culture of *R. eutropha* KCTC 2658 in Flask from VFAs

A batch culture was carried out to monitor the growth and PHA production kinetics of *R. eutropha* KCTC 2658 using 5 g/L VFAs as carbon source. Fig. 2(a) shows that butyric acid was consumed in the first 20 h, propionic acid was consumed continuously, but acetic acid was not depleted during 72 h incubation period. Over time, DCW and PHA concentration increased to a maximum of 1.8 g/L and 0.82 g/L at 30 h. The content of 3HB was reduced with culture time; accordingly the proportion of 3HV content increased to a maximum of 23 mol% (Fig. 2(b)). It is well known that P(3HB-co-3HV) copolymers are produced in *R. eutropha* cells when odd-number fatty acids such as propionic and valeric acids are added as carbon sources, while even-number fatty acids such as acetic and butyric acids produce only P(3HB) homopolymer [1]. This is consistent with the results in Fig. 2, showing that 3HV fraction started to increase with consumption of propionic acid. On the basis of the results obtained in flask cultures, batch and fed-batch cultures were carried out in a 5 L fermentor to investigate the kinetics of P(3HB) and P(3HB-co-3HV) production in more detail.

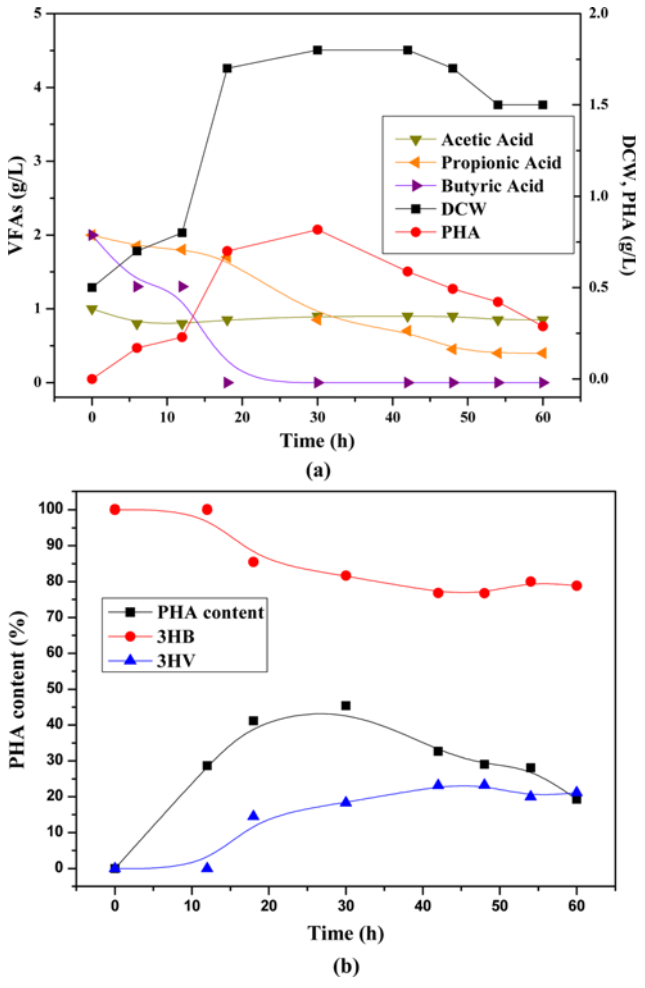

Fig. 2. Batch culture of *R. eutropha* KCTC 2658 in 250 mL flask. The graphs depict time courses of (a) dry cell weight, PHA concentration, and the concentrations of VFAs (acetic, propionic, and butyric acids) and (b) PHA content and copolymer composition following an initial VFAs concentration of 5 g/L (acetic acid : propionic acid : butyric acid=1 : 2 : 2).

4. Batch and Fed-batch Cultures of *R. eutropha* KCTC 2658 in Fermentor from Glucose and VFAs

Because VFAs are inhibitory and toxic to the bacterium, both cell growth and PHA production were limited in batch culture from VFAs as carbon source. Therefore, a cultivation strategy was suggested where cells were first grown on glucose and VFAs were supplied after glucose exhaution. First, batch culture of *R. eutropha* KCTC 2658 was carried out in 5 L fermentor using 20 g/L glucose as carbon source (Fig. 3). The glucose concentration gradually decreased. The highest DCW and PHA concentration were 8.4 g/L and 2.5 g/L, respectively, at 33 h. The PHA content was 30%, which was lower than from VFAs. Only P(3HB) homopolymer was produced from glucose as carbon source.

To improve the production of PHA, two fed-batch cultures were carried out. After cells were grown on 20 g/L glucose, VFAs were added using two feeding strategies. Fig. 4 shows the results of fed-batch culture with pulse feeding strategy. DCW and PHA concentration increased with time, while glucose and VFAs concentration declined. After feeding of VFAs at 38 h, DCW increased to 10.6 g/L at 58 h and then decreased with the consumption of VFAs. PHA concentration also increased to 3.5 g/L in accordance with DCW, resulting in a PHA content of 33%. As in flask batch culture using VFAs (Fig. 2), VFAs consumption was on the order of butyric acid,

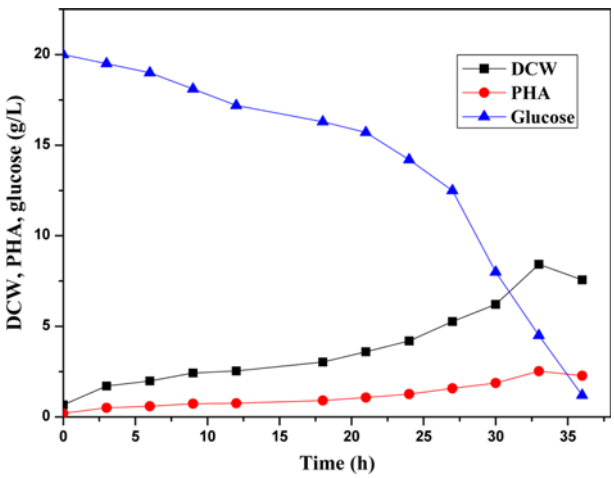

Fig. 3. Batch culture of *R. eutropha* KCTC 2658 in 5 L fermentor from 20 g/L of glucose as carbon source.

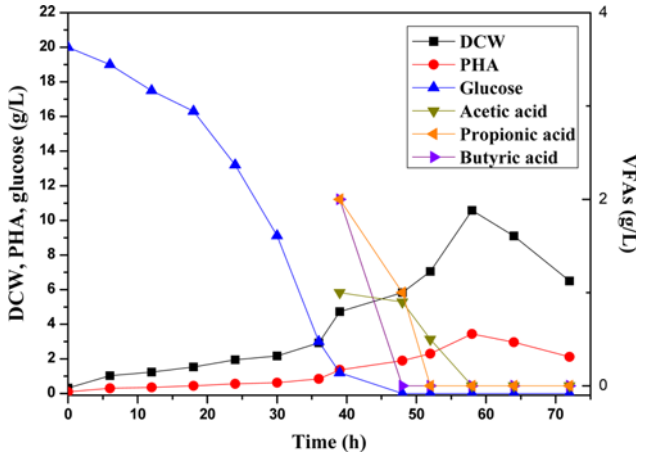

Fig. 4. Fed-batch culture of *R. eutropha* KCTC 2658 in 5 L fermentor with pulse feeding strategy. Cells were first grown using 20 g/L glucose and 5 g/L VFAs were fed at 38 h.

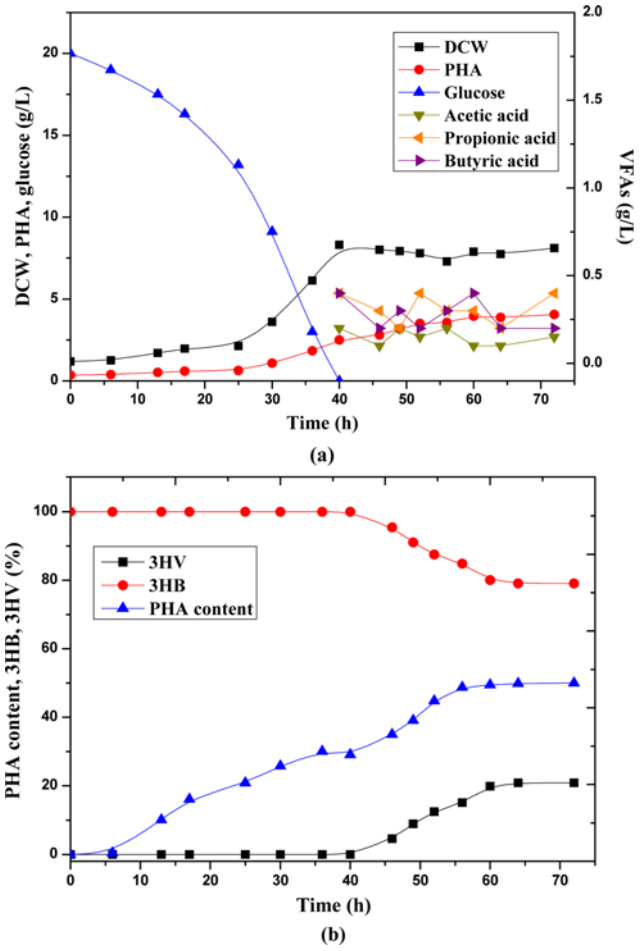

Fig. 5. Fed-batch culture of *R. eutropha* KCTC 2658 in 5 L fermentor with pH-stat feeding strategy. The graphs depict time courses of (a) dry cell weight, PHA concentration, and the concentrations of glucose and VFAs (acetic, propionic, and butyric acids) and (b) PHA content and copolymer composition. Cells were first grown using 20 g/L glucose and VFAs were automatically added by acid-supplying pump when pH rose above 6.5 after 40 h.

propionic acid, and acetic acid. Although a small amount of propionic acid (total 2 g/L) was added, the 3HV fractions in polymer samples were under the detectable range in the GC chromatogram due to relatively low concentration of propionic acid and low conversion efficiency of propionic acid to 3HV unit. Most carbon sources (20 g/L glucose, 2 g/L butyric acid, and 1 g/L acetic acid) were converted to the 3HB unit in PHA as well as cell growth and only small portion (2 g/L propionic acid) was converted to 3HV unit. In batch culture using 5 g/L VFAs (Fig. 2), however, a relatively large supply of propionic acid (2 g/L) as carbon source (total 5 g/L VFAs) produced copolymer containing 23 mol% 3HV. The conversion efficiency of propionic acid to the 3HV unit in PHA is known to be lower than that of valeric acid. The 3HV fractions in the copolymer produced by *R. eutropha* were 40 mol% from propionic acid and 90 mol% from valeric acid as a sole carbon source, respectively [1,7].

The results of fed-batch culture with pH-stat feeding strategy are shown in Fig. 5(a) and (b). When initially supplied glucose was consumed, the pH increased. By automatic feeding of mixed VFAs using acid pump for pH control, all VFAs were well maintained in culture broth during the fed-batch period of cultivation. DCW and PHA concentration increased to 8.1 g/L and 4.1 g/L, respectively, at 72 h. The PHA content was 50%, which was higher than that with pulse feeding strategy. Besides, 3HV fraction in the copolymer increased to 20 mol% due to the maintenance of propionic acid in culture broth.

CONCLUSIONS

Among the six strains tested, *R. eutropha* KCTC 2658 accumulated the highest amounts of PHAs, up to 50% of DCW from total 5 g/L of VFAs (acetic acid : propionic acid : butyric acid=1 : 2 : 2) as carbon source (Table 1). Cell growth decreased with increasing the initial concentration of VFAs. Batch culture kinetics showed that butyric acid was consumed first with cell growth and then propionic acid was consumed, resulting in an increase in 3HV fraction. With glucose as carbon source, homopolymer of 3HB was produced with PHA content of 30%. By automatic feeding of mixed VFAs using pH-stat feeding strategy, copolymer of 3HB and 3HV with 20 mol% 3HV was produced up to 50% of DCW. This work shows that VFAs generated from the acid hydrolysis of high strength organic food waste or waste sludge, such as butyric acid, propionic acid, and acetic acid, can be used for the bacterial biosynthesis of PHA by the *R. eutropha* KCTC2658. This process has the potential to reduce the cost of PHA production while also offering environmental benefits through the reuse of food trash and waste.

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