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Production of polyhydroxyalkanoates by batch and fed-batch cultivations of *Bacillus megaterium* from acid-treated red algae

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Abstract−Polyhydroxyalkanoates (PHAs) are linear polyesters synthesized by microbial fermentation of various substrates. PHAs are accumulated in microbial cells in order to store carbon and energy for future use. We used acid-pretreated red alga (*Gelidium amansii*) as a cheap, abundant carbon source to produce PHA via batch and fed-batch cultivation of *Bacillus megaterium* KCTC 2194. After acid treatment of 10% (w/v) *G. amansii*, 25.5 g/L galactose, 3.6 g/L glucose, 6 g/L 5-HMF, and 1.05 g/L levulinic acid were formed. In batch culture at pH 7, the dry cell weight (DCW) and PHA content increased to 5.5 g/L and 51.4%, respectively. The cell concentration was enhanced by fed-batch cultivation using two feeding strategies: pH-stat and intermittent feeding. When the pH-stat feeding strategy was employed to add concentrated hydrolysate to the fermentor, DCW increased to 8.2 g/L, with 53.2% PHA content. When concentrated hydrolysate was fed using the intermittent feeding strategy, higher DCW (10.1 g/L) was obtained, along with a slight increase of PHA content to 54.5%. This study demonstrates that red algae could be used after simple acid treatment, to produce PHA without steps for enzymatic hydrolysis and inhibitor removal.

Keywords: Polyhydroxyalkanoates, PHA, Red Algae, *Gelidium amansii, Bacillus megaterium*, Fed-batch Cultivation

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible polyesters that can substitute for petroleum-based thermoplastics. PHAs have properties similar to the latter, but are more easily biodegradable under a variety of environmental conditions [1]. The commercial viability of microbial PHA undoubtedly depends on the cost of the production process and the properties of the polymers produced [2,3]. Profitable commercial production of PHA requires using relatively cheap substrates such as volatile fatty acids [4], starch [5], whey [6], and sugar cane molasses [7] as sole carbon source. These carbon sources have been widely employed for PHA biosynthesis using several microorganisms (e.g., *Cupriavidus necator*, *Haloferax mediterranei*, *Bacillus megaterium*, and recombinant *Escherichia coli* strains). Many researchers are now investigating the potential of cheaper substrates for PHA production.

The red alga *Gelidium amansii* has attracted attention recently due to its high carbon content and availability. It grows faster than land plants and can be cultivated in vast areas of the ocean without nitrogen-based fertilizers [8]. In addition, *G. amansii* does not contain lignin. Without the expensive steps required to remove lignin, mono-sugar production becomes much cheaper [9]. This red alga is generally composed of cellulose and galactan. Hydrolysis of *G. amansii* produces major mono-sugars including D-galactose; 3,6-anhydro-L-galactose (3,6-AHG); and D-glucose [10]. Among these mono-sugars, only D-galactose and D-glucose are fermentable sugars released by enzymatic and acidic hydrolysis treatments of *G. amansii* [11]. Because 3,6-AHG is acid-labile, it decomposes to 5-hydroxymethylfurfural (5-HMF), levulinic acid, and formic acid, which inhibit the fermentation process [10]. Species of the genus *Bacillus* were among the first reported bacteria that could produce PHA, and have the ability to grow rapidly while utilizing a variety of complex carbon sources including sugarcane molasses, whey, wheat bran, and fruit industry wastes [12]. Among *Bacillus* species, *B. megaterium* appears exceptional in that the presence of this bacteria in soil has been linked to degradation of complex carbon sources and accumulation of PHA [12,13]. *B. megaterium* is a Gram-positive, aerobic, spore-forming species that lacks a lipopolysaccharide (LPS) layer. This makes the PHA separation process cheaper and easier than with a Gram-negative bacterial species [14,15]. In addition, the presence of endotoxins in Gram-negative bacteria induces strong immunogenic reactions, which is strictly prohibited for biomedical applications of PHA [15]. Recently, *B. megaterium* has been recognized as a promising bacterial candidate to produce PHA in large-scale fermentation due to its ability to utilize complex, cheap carbon sources [16]. Employing *B. megaterium* for industrial production of PHA would have several advantages, including cheap minimal medium requiring no growth factor, absence of endotoxin production, and high PHA accumulation [17]. Although *G. amansii* has been suggested as an interesting and potentially low cost carbon source for biofuel production [9], PHA production from *G. amansii* has not been reported other than in our previous study [18]. We screened six *B. megaterium* strains obtained from the Korean Culture Type Collection for their ability to accumulate PHA using acid-treated and untreated *G. amansii* as a carbon source [18]. Among the tested strains, *B. megaterium* KCTC 2194 was selected because its PHA content was the highest. In this study, we enhanced PHA production using acid-treated *G. amansii* by batch and fed-batch

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cultures of *B. megaterium* KCTC 2194 in a 2.5 L fermentor.

MATERIALS AND METHODS

1. Microorganism

Bacillus megaterium KCTC 2194 was obtained from the Korean Collection for Type Cultures (KCTC). The strain was activated in Luria Broth (LB) medium consisting of peptone 10 g/L, yeast extract 5 g/L, and NaCl 5 g/L; then was stored with glycerol (25% v/v) in 1.5 mL micro tubes at −20 °C, for future use.

2. Medium Preparation

LB medium was used for seed culture. A minimal medium stock was prepared: $(NH_4)_2SO_4$ 1.66 g/L, KH_2PO_4 2.16 g/L, $Na_2HPO_4 \cdot 12H_2O$ 8.64 g/L, $MgSO_4 \cdot 7H_2O$ 0.3 g/L, and 1 mL/L of trace element solution. 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, $(NH_4)6Mo_7O_{24} \cdot 4H_2O$ 0.03 g/L, $NiCl_2 \cdot 6H_2O$ 0.02 g/L, and $CuSO_4 \cdot 5H_2O$ 0.01 g/L. The seaweed powder was prepared as described previously [11]; *G. amansii* leaves were dried in the oven at 60 °C and then ground in a blender. The resulting powder was further prepared by passage through a 200-mesh sieve and then subjected to acidic treatment. A total of 10% (w/v) slurry content of *G. amansii* was treated with 94 mM H_2SO_4 at 121 °C for 60 min. The pH was fixed at 7 using 2 N NaOH prior to analysis with high-performance liquid chromatography (HPLC). A total of 10 mL of the minimal medium stock was mixed with 25 mL of distilled water and 25 mL of the hydrolysate *G. amansii*, resulting in the inoculum medium containing 12.1 g/L total sugar (10.6 g/L galactose and 1.5 g/L glucose). To investigate the optimum initial concentration of the hydrolysate, 10 mL of the minimal medium stock was added to different volumes of the hydrolysate *G. amansii* and distilled water, resulting in final media containing different concentrations of total sugar from 9.7 to 21.9 g/L. For the batch and fed-batch cultures in the fermentor, the initial minimal medium consisted of 9.7 g/L of total sugar (8.5 g/L galactose and 1.2 g/L glucose) released from acid-treated *G. amansii*, as well as $(NH_4)_2SO_4$ 1 g/L, KH_2PO_4 3.6 g/L, $Na_2HPO_4 \cdot 12H_2O$ 14.4 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, and 5 mL/L of trace element solution.

3. Bacterial Culture Conditions

B. megaterium KCTC 2194 was grown in a 250 mL flask containing 50 mL of LB medium at 30 °C, 175 rpm for 24 h. A total of 1 mL was inoculated into 100 mL of the inoculum medium, and then further incubated at 30 °C and, 175 rpm for 24 h. A 10% (v/v) portion of the inoculum culture was transferred into a 2.5 L stirred fermentor (KoBio Tech, Korea). The initial working volume was 750 mL. The culture temperature, pH, and aeration rate were maintained at 30 °C, 7, and 1.0 vvm, respectively. The pH was maintained with 5 N NaOH and 5 N HCl. For the fed-batch culture, the hydrolysate *G. amansii* was concentrated using a rotary evaporator to obtain a 10-fold concentrated feeding solution containing ~270 g/L of total sugar. For the pH-stat fed-batch culture, the feeding solution was prepared by mixing two volumes of the concentrated hydrolysate with one volume of 6 N NaOH, resulting in 2 N NaOH containing ~180 g/L of total sugar. The resulting alkaline solution was used for feeding and maintaining the pH. The intermittent feeding fed-batch culture was initially carried out in batch mode as described above, and the concentrated hydrolysate was directly used for feeding after 18 h cultivation, with an equal amount (8 mL, containing total galactose and glucose amount of 2.15 g) every 6 h. The feeding was continued until the dry cell weight (DCW) and PHA concentration stopped increasing.

4. Analytical Methods

Biomass was determined by measuring the DCW. The fermentation broth sample (10 mL) was centrifuged and cells were washed twice with distilled water; then further dried using a vacuum dryer at −51 °C for 24 h. The supernatant was collected for further sugar analysis. Glucose, galactose, 5-HMF, and levulinic acid concentrations were measured using an HPLC system (YL 9100, YOUNGLIN, Inc., Korea) using a Biorad Aminex hpx-87h column with a refractive index detector. The PHA content (% w/w PHA/dry cell) was determined using gas chromatography (GC). A total of 9 to 11 mg of lyophilized biomass was mixed with 1 mL chloroform and 1 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 an oven at 100 °C for 5 h and then cooled to room temperature. Prior to analysis, 1 mL of HPLC water was added and mixed. After layer separation, the bottom phase was used for injection into a 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 the GC were injection volume=1 μL, 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.

RESULTS AND DISCUSSION

1. Effect of Initial *G. amansii* Concentration

We screened six *B. megaterium* strains for their ability to produce PHA from glucose and galactose, and from un-treated and acid-treated *G. amansii*. Among the tested strains, *B. megaterium* KCTC 2194 accumulated the most PHA (55%) from treated algae and from untreated algae (28%) [18]. The natural type of PHA was found to be poly(3-hydroxybutyrate) homopolymer by GC analysis. This study enhanced PHA production by investigating shaken-flask culture, batch culture with pH control, and fed-batch culture using concentrated *G. amansii* hydrolysate as a feeding solution.

B. megaterium KCTC 2194 utilizes the available sugars in the *G. amansii* hydrolysate medium (glucose and galactose), for both biomass and PHA production. Prior to the bioreactor experiments, the effect of initial inhibitor concentrations of *G. amansii* hydrolysate was determined in flask culture. After acid treatment of 10% (w/v) *G. amansii*, 25.5 g/L galactose, 3.6 g/L glucose, 6 g/L 5-HMF, and 1.05 g/L levulinic acid were formed. Six media with different concentrations of *G. amansii* hydrolysate were examined. During 24 h cultivation, *B. megaterium* KCTC 2194 showed an ability to grow in four samples, and the fastest growth occurred with the initial sugar concentration of 9.7 g/L and inhibitor concentrations of 2 g/L and 0.35 g/L for 5-HMF and levulinic acid, respectively (Fig. 1). Cell growth decreased as the initial inhibitor concentrations increased due to their negative effect on cell replication. This

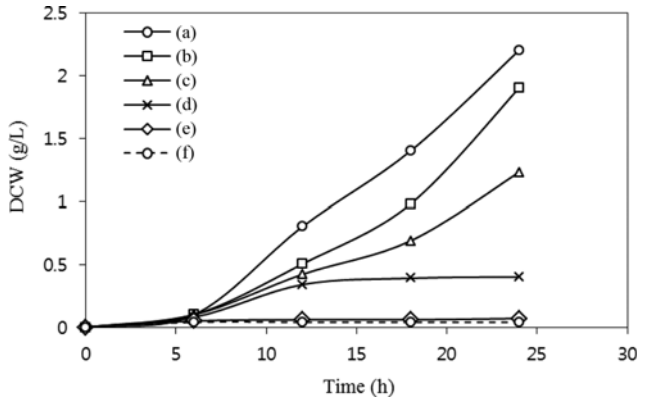

Fig. 1. Effect of different initial sugar and inhibitor concentrations on growth of *B. megaterium* KCTC 2194, under the conditions (a) 8.5 g/L galactose, 1.2 g/L glucose, 2 g/L HMF, and 0.35 g/L levulinic acid; (b) 12.1 g/L galactose, 1.5 g/L glucose, 2.5 g/L HMF, and 0.44 g/L levulinic acid; (c) 12.8 g/L galactose, 1.8 g/L glucose, 3 g/L HMF, and 0.52 g/L levulinic acid; (d) 14.9 g/L galactose, 2.1 g/L glucose, 3.5 g/L HMF, and 0.61 g/L levulinic acid; (e) 17.1 g/L galactose, 2.4 g/L glucose, 4 g/L HMF, and 0.7 g/L levulinic acid; (f) 19.3 g/L galactose, 2.7 g/L glucose, 4.5 g/L HMF, and 0.8 g/L levulinic acid.

occurred via induction of increased lag-phase growth and a significant drop in intracellular pH [19]. Therefore, higher concentrations of initial sugar and inhibitors resulted in cell-growth suppression. A total growth inhibition occurred at inhibitor concentrations in the medium of higher than 4 g/L of 5-HMF and 0.7 g/L of levulinic acid. These results suggested that the best initial *G. amansii* hydrolysate solution to be employed for large-scale PHA production would be a medium containing 1.2 g/L glucose, 8.5 g/L galactose, 2 g/L 5-HMF, and 0.35 g/L levulinic acid.

2. PHA Production in Flask Culture

A batch culture in a 1 L flask with working volume of 200 mL was carried out to investigate the growth and PHA production kinetics of *B. megaterium* KCTC 2194. This was done in hydrolysate medium containing 1.2 g/L glucose, 8.5 g/L galactose, 2 g/L of 5-HMF, and 0.35 g/L of levulinic acid, released from acid-treated *G. amansii* (Fig. 2(a)). During the cultivation of *B. megaterium* KCTC 2194, we found that the strain utilized glucose for the first 12 h and then continuously consumed galactose, which could be attributed to the preference for glucose over galactose. Concentrations of 5-HMF and levulinic acid were almost constant at 2 g/L and 0.35 g/L, respectively, during 48 h of cultivation. The pH decreased from 7 to 5.5 after 48 h of cultivation. The PHA content at 42 h was 49% with 3.3 g/L of DCW, and both PHA content and DCW were slightly decreased at the end of the cultivation. On the other hand, *B. megaterium* KCTC 2194 was cultivated in minimal medium containing 1.2 g/L glucose and 8.5 g/L galactose as a positive control, and 1.2 g/L glucose, 8.5 g/L galactose, 2 g/L 5-HMF, and 0.35 g/L levulinic acid as a negative control. Fig. 2(b) and (c) show that 5-HMF and levulinic acid in the negative control slightly suppressed cell growth and PHA accumulation, compared to the positive control, which started to produce PHA after 12 h.

3. PHA Production in Fermentor

Two bioreactor experiments were conducted using *G. amansii*

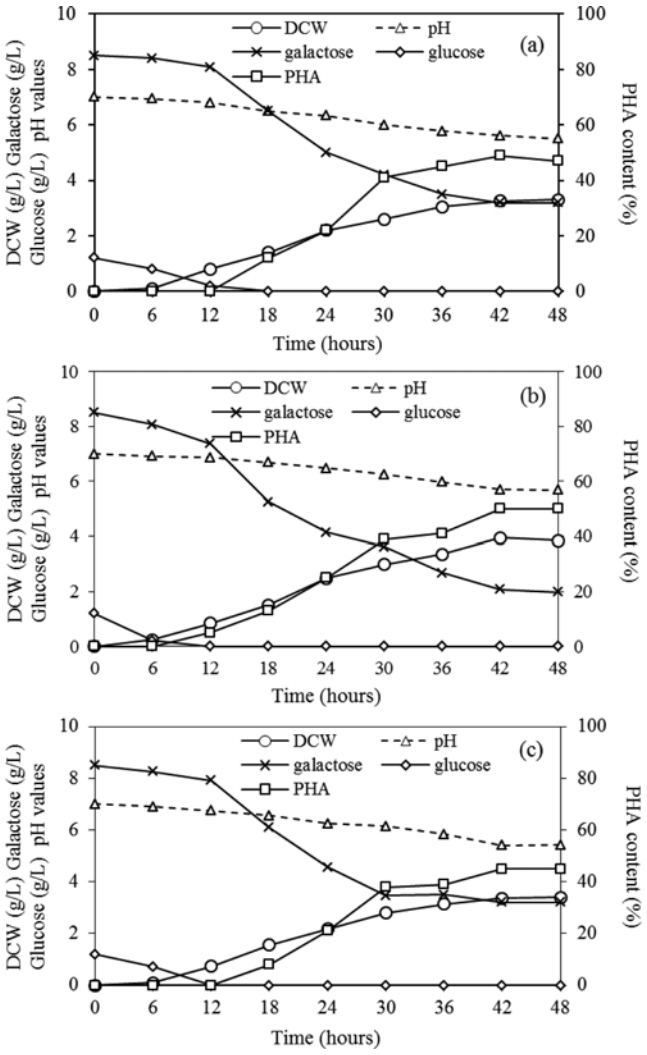

Fig. 2. Growth of *B. megaterium* KCTC 2194 in flask batch culture at 30 °C using 9.7 g/L sugar as a carbon source: (a) *G. amansii* hydrolysate containing 1.2 g/L glucose, 8.5 g/L galactose, 2 g/L HMF, and 0.35 g/L levulinic; (b) 1.2 g/L glucose and 8.5 g/L galactose as a positive control; (c) 1.2 g/L glucose, 8.5 g/L galactose, 2 g/L HMF, and 0.35 g/L levulinic acid as a negative control.

hydrolysate as a carbon source. The main purpose of these fermentation experiments was to identify the best cultivation conditions for increasing both DCW and PHA. A batch culture, in which pH 7 was maintained, was run with a working volume of 1 L in a 2.5 L fermentor (Fig. 3). The consumption of sugars (glucose and galactose), DCW, and PHA was monitored during 36 h of cultivation. Compared to the flask culture without pH control (Fig. 2), higher DCW (5.5 g/L) and PHA content (51.4%) were obtained by controlling the pH at 7 in the fermentor. The glucose present was completely utilized after 8 h cultivation and then cells started to consume galactose. Only traces of galactose remained in the medium after 36 h of cultivation.

For most PHA producers such as *C. necator*, *Bacillus cereus*, recombinant *E. coli*, and *B. megaterium*, higher PHA concentrations are usually achieved using fed-batch culture rather than batch cul-

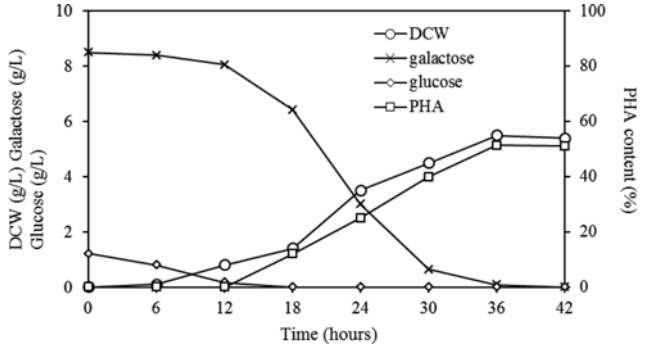

Fig. 3. Batch culture of *B. megaterium* KCTC 2194 at 30 °C using 9.7 g/L sugar (1.2 g/L glucose and 8.5 g/L galactose) as a carbon source in a 2.5 L fermentor with working volume of 750 mL.

ture [4,20-22]. To enhance DCW and PHA production, fed-batch cultures with two feeding strategies (pH-stat and intermittent feeding) were employed. Cells were grown in an initial working volume of 1 L in a 2.5 L fermentor. The pH-stat feeding was carried out by mixing the feeding solution with NaOH, resulting in 2 N NaOH containing concentrated *G. amansii* hydrolysate. According to the results from a previous study, a respectable decrease in the inhibitor 5-HMF concentration accompanied evaporation [10], which made *B. megaterium* KCTC 2194 grow better. Compared to batch culture, the pH-stat fed-batch culture yielded higher DCW (8.2 g/L) with a slight increase of PHA content (to 53.2%) after 48 h of cultivation (Fig. 4(a)). The growth then decreased slightly. This was probably due to accumulated inhibitors in the medium. DCW and PHA content were further increased using intermittent feeding, as shown in Fig. 4(b). The feeding was performed every 6 h using 8 mL of concentrated *G. amansii* hydrolysate, which contained total galactose and glucose amount of 2.15 g. At 60 h, DCW and PHA content were 10.1 g/L and 54.5%, respectively. Table 1 summarizes previous reports using *Bacillus* strains for PHA production from different complex carbon sources. Our results, using red algae as a cheap, abundant complex carbon source, are comparable to previous studies in terms of PHA content. According to Table 1, among the reported *Bacillus* strains, *B. megaterium* KCTC 2194 strain exhibited results in the high range of PHA content (51-54%). This is similar to *B. megaterium* MSBN04, which accumulated up to 58% PHA, using tapioca industry waste as a

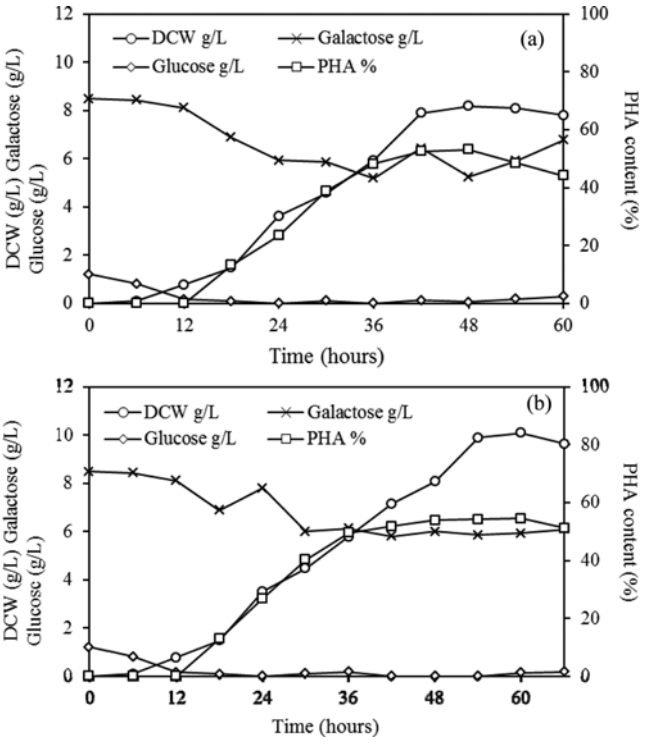

Fig. 4. Fed-batch culture of *B. megaterium* KCTC 2194 at 30 °C using 9.7 g/L sugar (1.2 g/L glucose and 8.5 g/L galactose) as initial carbon source in a 2.5 L fermentor with initial working volume of 750 mL. During fed-batch cultivation, a feeding solution containing concentrated *G. amansii* hydrolysate was fed into the fermentor using (a) pH-stat feeding, and (b) intermittent feeding after 18 h of batch cultivation.

carbon source [12]. The fed-batch cultivation technique is considered the best method for achieving a remarkable enhancement of DCW and PHA accumulation in bacterial cells and for yielding a high cell-density culture [23,24]. In our experiment, we could not achieve a high cell-density culture, due to increasing amounts of accumulated inhibitors in the medium like 5-HMF and levulinic acid. Although *B. megaterium* KCTC 2194 did not grow to very high cell density, the medium used in this study was prepared using a simple acid treatment without steps for enzymatic hydrolysis and inhibitor removal.

Table 1. Comparison of *Bacillus* strains producing PHA from various complex carbon sources

Microorganism	Carbon source	Cultivation technique	PHA content (%)	Reference
B. megaterium KCTC 2194	Red sea algae *G. amansii*	Flask, batch, fed-batch (pH-stat and intermittent feeding)	51-54	This work
B. megaterium SRKP-3	Dairy wastes, rice bran, sea water	Flask shake, fed-batch	6.4-11.3	[26]
B. megaterium	Sugarcane molasses	Batch	59	[7]
B. megaterium BA-019	Sugarcane molasses	Batch	27	[25]
B. megaterium BA-019	Sugarcane molasses	Fed-batch (intermittent feeding)	46	[25]
Bacillus sp. JMa5	Sugarcane molasses	Batch, fed-batch	25-35	[27]
B. subtilis NG220	Sugar industry waste water	Batch	51.8	[28]
B. megaterium MSBN04	Tapioca industry waste	Batch	58	[12]

CONCLUSIONS

This study demonstrated that a simple, cheap medium prepared quickly using acid-treated red algae (*G. amansii*), could be used to produce biopolymer PHA using the *B. megaterium* KCTC 2194 strain in batch and fed-batch cultures. Cell growth decreased with increasing initial sugar concentration due to the rise of inhibitor concentrations in the medium. *B. megaterium* KCTC 2194 could accumulate PHA up to 51-54% of its DCW. By fed-batch culture, DCW was enhanced to 10.1 g/L compared to those in flask (3.3 g/L) and batch culture (5.5 g/L). Glucose was consumed before galactose. Based on these findings, future studies can be performed with further hydrolysis of *G. amansii* to produce higher amounts of glucose, and removal of 5-HMF and other inhibitors from the medium prior to cultivation.

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