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CHARACTERISATION OF POLYHYDROXYALKANOATE PRODUCTION BY MUTANT *Azotobacter vinelandii*

MOK, PEI SHZE[1,2], SUDESH, KUMAR[2], LIEW, PAULINE WOAN YING[1*], JONG, BOR CHYAN[1] and NAJIMUDIN, NAZALAN[2]

[1]*Agrotechnology and Bioscience Division, Malaysian Nuclear Agency, Bangi, 4300 Kajang, Selangor, Malaysia*
[2]*School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia*
[*]*E-mail: paulineliew@nuclearmalaysia.gov.my*

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ABSTRACT

The production of PHA by *Azotobacter vinelandii* was characterised in different types of mineral medium including DSMZ-Azotobacter medium, Ashby's medium, Burk's medium and Minimal Medium PHA (MMPHA). A deletion mutant of *Azotobacter vinelandii*, designated as Δ*Avin_16040* was able to accumulate 3-folds higher PHA which was 0.48 ± 0.02 g/L compared to the wild type strain which was 0.11 ± 0.03 g/L in a modified DSMZ-Azotobacter medium. Although this bacterial strain is able to fix nitrogen, addition of urea as external nitrogen source into the medium had increased both its biomass and PHA production. Among the various medium types, MMPHA showed the highest biomass and PHA yields of 3.5 ± 0.1 g/L and 1.50 ± 0.03 g/L, respectively. Higher PHA concentration of 1.74 ± 0.05 g/L was obtained in this medium by increasing the sucrose concentration from 20 g/L to 25 g/L.

Key words: Polyhydroxyalkanoate, *Azotobacter vinelandii*, mutant, nitrogen, sucrose concentration

INTRODUCTION

Polyhydroxyalkanoate (PHA) is known as a biodegradable and natural thermoplastic that can be accumulated by various microbes such as *Azotobacter vinelandii, Azotobacter chroococcum*, *Cupriavidus necato*r, *Alcaligenes* spp., *Pseudomonas* spp., *Bacillus* spp., *Aeromonas hydrophila* and *Burkholderia sacchari*. It can be produced in the presence of an excess carbon source and limiting nutrients such as nitrogen, phosphorus, sulphur and oxygen. This polymer is stored as intracellular granules as microbes' carbon and energy sources (Steinbüchel *et al.*, 1992; Jendrossek & Pfeiffer, 2014; Prieto *et al.*, 2015). Due to the similar properties between PHA and synthetic petrochemical plastic such as polypropylene and polyethylene, it has the potential to replace the synthetic plastic in the future. One drawback is its high production cost as compared to that of synthetic plastics. Thus, one main interest is to reduce its cost such as by using renewable carbon source and modifying the downstream processes (Du *et al.*, 2012; Muhammadi *et al.*, 2015). There are currently more than 150 types of monomers that have been discovered. Poly(3-hydroxybutyrate) [P(3HB)] is the most common and first discovered PHA (Lemoigne, 1926; Keshavarz & Roy, 2010). This polymer is relatively abundant and can be found in various microorganisms. Its composition in microbial cell is low but can be increased to more than 90% by adjusting the fermentation conditions (Penloglou *et al.*, 2012).

A. vinelandii is a Gram-negative soil bacterium that can fix nitrogen. This strain has the ability to accumulate P(3HB) under unbalanced growth conditions (Page & Knosp, 1989). When carbon source is limited, this polymer will be used by the cell to turn into cyst. Previous studies showed that *A. vinelandii* UWD could accumulate up to 2.5 g/L of P(3HB) using glucose as carbon source. This mutant strain did not form capsule, thus limitation to produce P(3HB) had been reduced (Page & Knosp, 1989). Besides, sugar beet molasses could generate 6.8 g/L of P(3HB) (Page, 1990). By conducting fed-batch fermentation, approximately

* To whom correspondence should be addressed.

25 g/L of P(3HB) was produced (Page & Cornish, 1993) while a maximal concentration of 36 g/L of P(3HB) was produced using a two-stage cultivation (Chen & Page, 1997). *A. vinelandii* mutant OPNA strain was able to accumulate 27.3 g/L of P(3HB) under exponentially fed-batch cultures (García *et al.*, 2014). In this study, the cell growth and PHA accumulation of *A. vinelandii* wild type and mutant Δ*Avin_16040* strains were compared. Different media were used and other conditions including addition of nitrogen source and concentration of carbon source were altered to aim for larger amount of P(3HB). Although much work has been done on the wild type *A. vinelandii* ATCC 12837, this is the first report on PHA production by the mutant strain Δ*Avin_16040*.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study were *A. vinelandii* ATCC 12837 wild type and mutant Δ*Avin_16040* strains. The mutant strain was described previously by Liew *et al* (2015).

Comparison of dry cell weight and PHA production of wild type and mutant of *A. vinelandii*

In order to compare the cell growth and PHA accumulation of wild type and mutant strains, single colony of *A. vinelandii* wild type and mutant strains was separately inoculated into Burk's medium with 1% w/v of glucose as carbon source and incubated for 3 days at 30°C and 200 rpm (Liew *et al*., 2015). Burk's medium (BM) consisted of 0.8 g/L of K_2HPO_4, 0.2 g/L of KH_2PO_4, 0.2 g/L of $MgSO_4{\cdot}7H_2O$, 1.45 mg/L of $FeCl_3$, 0.253 mg/L of $Na_2MoO_4{\cdot}2H_2o$ and 0.13 g/L of $CaSO_4{\cdot}2H_2O$. One percent (v/v) of each bacterial culture was transferred to modified DSMZ-Azotobacter medium (MDA) containing 2% (w/v) of sucrose at an initial cell count of 10^5 colony forming unit per mL. Samplings were carried out at different durations starting from 24 hrs to 96 hrs. DSMZ-Azotobacter medium (DA) consisted of 0.9 g/L of K_2HPO_4, 0.1 g/L of KH_2PO_4, 0.01 g/L of $FeSO_4$, 0.1 g/L of $CaCl_2$, 0.1 g/L of $MgSO_4{\cdot}7H_2O$, 5.0 mg/L of $Na_2MoO_4{\cdot}2H_2o$ and 5.0 g/L of $CaCO_3$. However, the non-water-soluble $CaCO_3$ contributed partly to the bacterial dry cell weight thus complicating the methanolysis-gas chromatography analysis of PHB. As such, a modified DSMZ-Azotobacter medium (MDA) was formulated by excluding $CaCO_3$ from the medium composition.

Subsequently, a "high cell mass inoculation approach" was conducted aiming to shorten the culturing period of the slow-growing *A. vinelandii* while still maintained an average or better performances. For this methodology, three loopfuls of wild type and mutant cells were individually scraped from freshly grown pure bacterial cultures on solid medium and transferred into NR broth containing 2% (w/v) of sucrose as carbon source. The components of NR broth were 10 g/L of peptone, 2 g/L of yeast extract and 10 g/L of meat extract with an initial pH of 7.0. Both bacteria were grown at 30°C under agitation of 200 rpm for 15 hrs. Three percent (v/v) of each culture was transferred into DSMZ-Azotobacter medium containing 2% (w/v) of sucrose to an initial cell count of 10^6 colony forming unit per mL. As before, samplings were carried out at different time durations starting from 24 hrs to 96 hrs. All the experiments were conducted in triplicates.

Optimisation of starter culture

A. vinelandii mutant was streaked on Nutrient Rich (NR) agar plate and incubated for 3 days at 30°C. To determine the most optimum inoculum, NR cultures of 12, 15, 18, 21 and 24-hr incubation periods were separately transferred to BM medium at 3% (v/v) concentration. Dry cell weight and PHB content was determined after 48 hrs of cultivation period as described before.

Evaluation of dry cell weight and PHA production by *A. vinelandii* mutant in various mineral media

A starter culture of *A. vinelandii* mutant was generated by adapting the "high cell mass inoculation approach" as described above. Nine mineral medium compositions including the derivatives of Ashby's medium (AM), Burk's medium (BM), DSMZ-Azotobacter medium (DA) and Minimal Medium PHA (MMPHA) were tested. AM consisted of 0.2 g/L of K_2HPO_4, 0.2 g/L of NaCl, 0.1 g/L of K_2SO_4, 0.2 g/L of $MgSO_4{\cdot}7H_2O$ and 5 g/L of $CaCO_3$ (Ashby, 1907). Modified AM (MAM) medium consisted of all the components of AM medium excluding $CaCO_3$. MMPHA consisted of 4.6 g/L of Na_2HPO_4, 4 g/L of NaH_2PO_4, 0.45 g/L of K_2SO_4, 0.062 g/L of $CaCl_2$, 0.39 g/L of $MgSO_4$ and 1 mL/L of trace elements (TE). TE consisted of 15 g/L of $FeSO_4{\cdot}7H_2O$, 2.4 g/L of $MnSO_4{\cdot}H_2O$, 2.4 g/L of $ZnSO_4{\cdot}7H_2O$ and 0.48 g/L of $CuSO_4{\cdot}5H_2O$ in 0.1 M of HCl (Budde *et al.*, 2010). The components of BM and DA media have been described in the previous section. AM, BM and DA media were commonly used for growth of *A. vinelandii* while MMPHA was manipulated for PHA production. All the mineral media were adjusted to pH 7.0 by adding 1 M of HCl or 1 M of NaOH. The seed culture in NR medium was transferred into the respective mineral medium and supplemented with 2% (w/v) of sucrose as carbon source. The bacterial

strain was grown at 30°C under agitation of 200 rpm. The cultures were harvested after incubation for 48 hrs. To characterise the production of P(3HB) in the presence of nitrogen, 0.54 g/L of urea was added to all the mineral media. The P(3HB) yields in the presence and absence of nitrogen source was compared. The effect of sucrose concentrations was evaluated in the MMPHA medium supplied with 0.54 g/L urea. For this purpose, sucrose concentrations of 5, 10, 15, 20 and 25 g/L were tested.

PHB analysis by methanolysis-gas chromatography method

The harvested cell was lyophilised via freeze-drying and the dry cell weight was obtained. In order to measure the PHA content and its composition in dry cell, gas chromatography (GC) was carried out using gas chromatography Shimadzu GC-2010 with AOC-20i Auto Injector (Braunegg *et al.*, 1978). Around 15 – 20 mg of lyophilised cells were weighed and subjected to methanolysis with methanol: sulfuric acid of 85:15 (v/v). The resulting hydroxyacyl methyl esters were then analysed and calculated based on standard methods.

RESULTS AND DISCUSSION

Comparison of dry cell weight and PHA production of wild type and mutant of *A. vinelandii*

Generally, the dry cell weight and PHA yields were higher for the mutant strain compared to the wild type strain (Figure 1). The dry cell weight of both wild type and mutant strains increased gradually and reached stationary phase after 81 hrs of cultivation. An average yields of 1.6 ± 0.1 g/L of dry cell weight and 0.11 ± 0.03 g/L (7 ± 2% cell content) of P(3HB) was obtained from the wild type strain while 2.2 ± 0.1 g/L of dry cell weight and 0.48 ± 0.02 g/L (23 ± 1% cell content) of P(3HB) was obtained from the mutant strain. The P(3HB) production by mutant strain was three folds higher than the wild type strain.

On the other hand, the "high cell mass inoculation approach" showed the mutant strain yielded slightly higher P(3HB) concentration of 0.68 g/L with 45% cell accumulation despite its lower dry cell weight of 1.5 g/L (Figure 2). Based on the higher P(3HB) yield and % P(3HB) content in cell, the "high cell mass inoculation approach" was employed to further explore the P(3HB)

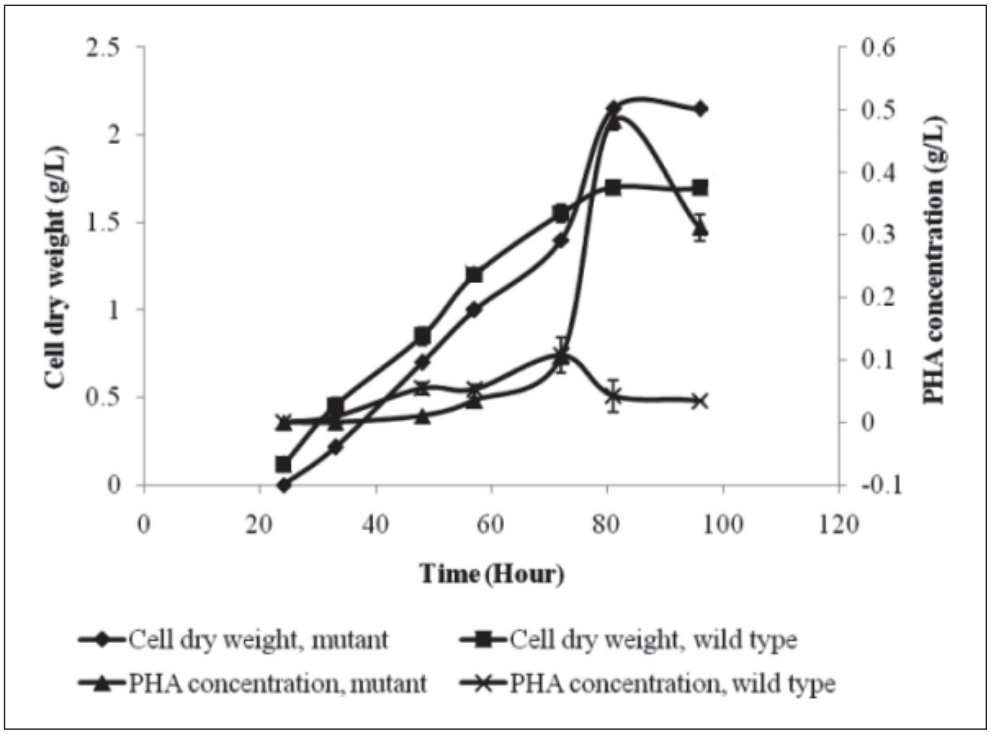

Fig. 1. Dry cell weights and PHA concentrations obtained from *A. vinelandii* ATCC 12837 wild type and mutant Δ*Avin_16040* strains. Starter culture was prepared by inoculating single cell colony into BM medium. Bacterial cells were cultured in MDA medium devoid of calcium carbonate at 30°C, 200 rpm.

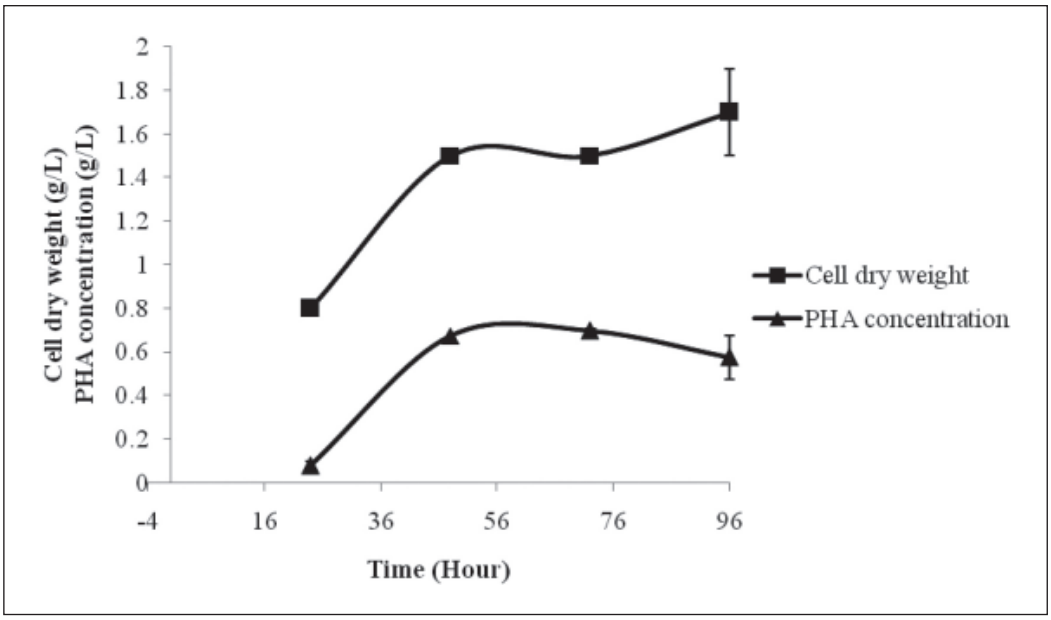

Fig. 2. Dry cell weights and PHA concentrations obtained from *A. vinelandii* mutant Δ*Avin_16040* strain. Starter culture was prepared according to the "high cell mass inoculation approach" and inoculated into BM medium. Bacterial cells were cultured in MDA medium devoid of calcium carbonate at 30°C, 200 rpm.

production by *A. vinelandii* mutant in various mineral media. Other research groups have used similar "high cell mass inoculation approach" to shorten the incubation period of inoculum as well as the incubation periods in the production medium. Butt *et al* (2011) inoculated loopful of *A. vinelandii* mutant which were grown for 24 hrs before it was transferred to the alginate production medium at 4% (v/v) inoculum concentration. Dhanasekar *et al* (2001) also transferred overnight culture of *A. vinelandii* MTCC 124* into P(3HB) production medium at 4% (v/v) inoculum concentration and grown for 48 hrs when the culture reached stationary growth phase with 1.48 g/L dry cell weight and 1.3 g/L P(3HB). The effect of inoculum sizes on cell growth and PHB production in batch culture of *A. vinelandii* was elaborated by Dhanasekar *et al* (2001). The *A. vinelandii* mutant grown using the "high cell mass inoculation approach" reached stationary faster than that of the "single colony method" possibly due to the inhibitory effect of glycine. Glycine was proved to interfere with peptidoglycan synthesis and could inhibit the cell growth (Hammes *et al.*, 1973; Minami *et al.*, 2004). This component could be found in the peptone of NR broth (Lundgren *et al.*, 2013).

Optimisation of starter culture

A. vinelandii mutant which was grown for a different period of times in NR medium was evaluated for use as the starter (inoculum) culture for its P(3HB) production. Based on the summarized results in Table 1, *A. vinelandii* mutant showed higher biomass and P(3HB) yields when BM medium was inoculated with 15-hr starter culture compared to 12-hr culture with the differences of 1-fold and 1.73-fold, respectively. Besides, PHB content in cell was higher by 37% in the production medium inoculated with 15-hr starter culture. Inoculation with the older starter cultures of 18-hr, 21-hr and 24-hr were not different from that of 15-hr. Thus, the 15-hr starter culture was used for

Table 1. Dry cell weight and PHA content of *A. vinelandii* mutant inoculated with inoculum of different cultivation periods

Inoculum cultivation period (hr)	Dry cell weight (g/L)	PHA content (%)	PHA concentration (g/L)
12	0.5 ± 0	41 ± 3	20.5 ± 1.4
15	1.0 ± 0	56 ± 1	56.0 ± 1.4
18	0.85 ± 0.07	58 ± 2	48.8 ± 2.3
21	0.95 ± 0.07	55 ± 4	51.7 ± 0.5
24	0.85 ± 0.07	51 ± 3	43.3 ± 1.2

Table 2. Dry cell weight and PHA content of *A. vinelandii* mutant Δ*Avin_16040* strain by cultivating them in AM, MAM, DA, MDA, BM and MMPHA media with or without nitrogen source

Medium	Addition of $CaCO_3$	Addition of urea	Dry cell weight (g/L)	PHA content (%)	PHA concentration (g/L)
AM	Yes	Yes	2.6 ± 0.2	50 ± 1	1.30 ± 0.28
AM	Yes	No	0.2 ± 0	28 ± 8	0.06 ± 0.03
MAM	No	Yes	2.1 ± 0.2	71 ± 1	1.48 ± 0.14
DA	Yes	Yes	2.5 ± 0.1	33 ± 2	0.83 ± 0.06
DA	Yes	No	2.3 ± 0	22 ± 3	0.49 ± 0.05
MDA	No	Yes	2.6 ± 0.2	5 ± 1	0.12 ± 0.04
BM	–	Yes	1.8 ± 0.1	42 ± 3	0.78 ± 0.09
BM	–	No	0.6 ± 0	34 ± 1	0.21 ± 0.01
MMPHA	–	Yes	3.5 ± 0.1	43 ± 0	1.50 ± 0.03

The mutant cells were cultured in Ashby's (AM), Burk's (BM), DSMZ-Azotobacter (DA) and minimal medium PHA (MMPHA) at 30ºC, 200 rpm for 48 hours. Sucrose of 2% (w/v) and urea of 0.54 g/L concentrations were added as carbon and nitrogen source respectively. MAM and MDA are modified AM and DA which was devoid of calcium carbonate.

subsequent works according to the "high cell mass inoculation approach".

Production of dry cell weight and P(3HB) by *A. vinelandii* mutant in various mineral media added with urea

The production of dry cell weight and P(3HB) in various media was summarized in Table 2. In general, addition of 0.54 g/L of urea increased the dry cell weight in all the medium compositions including AM, MAM, DA, MDA and BM media. Its effect was not obvious for DA and MDA. In the media devoid of nitrogen, DA showed the highest dry cell weight of 2.3 ± 0 g/L. The other N-free media demonstrated low cell biomass of 0.2 (AM) and 0.6 g/L (BM) dry cell weights. The higher dry cell weight as obtained from DA medium lacking nitrogen suggested that $FeSO_4$ and $NaMoO_4$ are important components in the medium that promoted the growth of *A. vinelandii* mutant cell. Iron ions play critical role in maintaining the level of superoxide dismutase that determines the viability of mutant cells (Kurutas, 2016; Maier & Moshiri, 2000) while Na_2MoO_4 was commonly used to promote the growth of difficult bacteria. The MMPHA medium represented the highest dry cell weight yield of 3.5 ± 0.1 g/L. *A. vinelandii* is a nitrogen-fixing bacterium in which it can fix atmospheric nitrogen for its own consumption (Ueda *et al.*, 1995). The process of fixing N_2 is a high energy-consuming mechanism. Therefore, when *A. vinelandii* mutant was provided with ready-to-use nitrogen source, it could have terminated or slowed down its N_2 fixation machineries, thereby conserved more energy (from carbon source utilisation) for biomass and PHB productions.

In the case of PHB production, all the media containing urea showed improved P(3HB) concentration and cell content except for MDA medium. Although the MDA medium produced no obvious change in the dry cell weight from DA medium, the P(3HB) yield observed selective reduction by 75% from 0.49 ± 0.05 g/L (22 ± 3% cell content) in DA to 0.12 ± 0.04 g/L (5 ± 1% cell content) in MDA. No such effect was observed in the AM medium which also consisted of 5 g/L of $CaCO_3$ as the DA medium. The low PHB yield in MDA medium was possibly caused by the absence of $CaCO_3$ in coincidence with the presence of urea. Since $CaCO_3$ (lime) has neutralising effect on soil humic acid, it may also have buffering function on the metabolic wastes from urea metabolism, minimizing any detrimental effect. Otherwise, the high Ca^{2+} in $CaCO_3$ (5 g/L) may enhanced the cell turgor, thus protecting the cell wall from denaturation by urea. Despite having reduced P(3HB) yield, the presence of urea in the MDA medium lacking $CaCO_3$ was able to maintain the growth of *A. vinelandii* mutant at the same level as DA medium (with and without urea). In general, nitrogen is the building block of living organisms. MMPHA was previously used to cultivate *Ralstonia eutropha* (Budde *et al.*, 2010). It was consisted of a variety of trace elements including manganese, copper and zinc salts that could stimulate cell growth and PHB production. In this medium, *A. vinelandii* mutant yielded 1.5 ± 0.03 g/L of P(3HB), similar to its P(3HB) yields in the AM and MAM media containing urea.

Effect of sucrose concentration

MMPHA medium was used to study the effect of sucrose concentrations on dry cell weight and PHB yields. According to Figure 3, accumulation of PHA was negligible when only 0.5% (w/v) of sucrose was added as carbon source. The low sucrose concentration was barely enough for *A. vinelandii* mutant to generate cell biomass (1.3 ± 0.07 g/L). By increasing sucrose concentration to 1.0% (10 g/L)

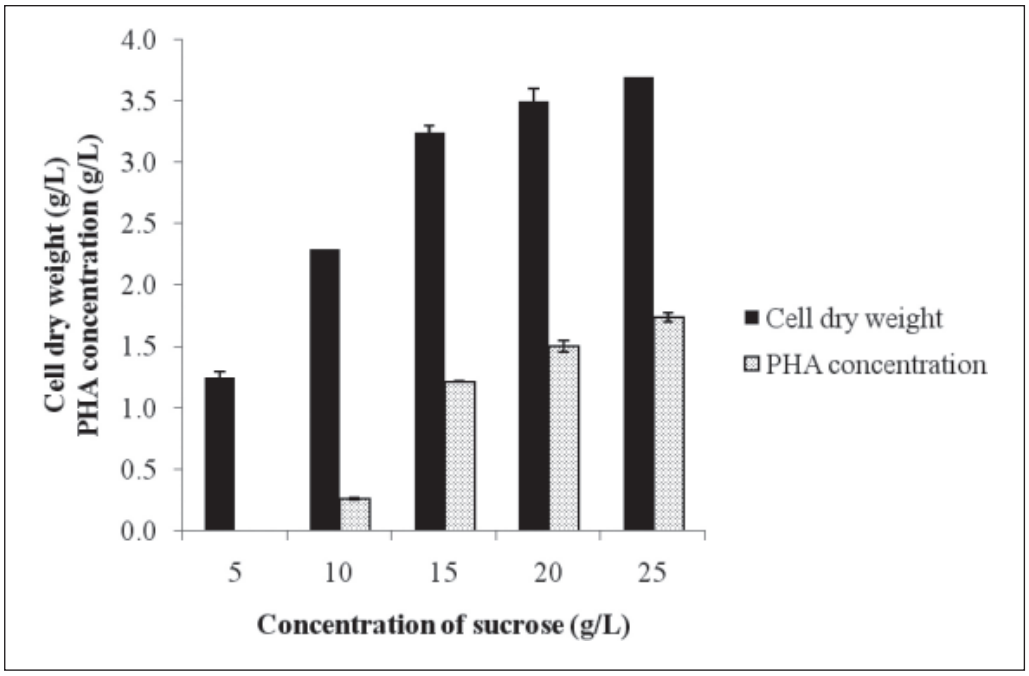

Fig. 3. Dry cell weight and PHA accumulation by *A. vinelandii* mutant Δ*Avin_16040* strain provided with different concentrations of sucrose. The cells were cultured in MMPHA at 30°C, 200 rpm for 48 hrs. Its cell biomass and PHA yields increased drastically when sucrose concentration was increased from 10 to 15 g/L. Subsequent addition in sucrose concentration caused very slight increments in cell biomass and PHA concentration.

the *A. vinelandii* mutant started to accumulate low P(3HB) concentration of 0.26 ± 0.02 g/L, then increased to 1.2 ± 0.05 g/L with 1.5% (15 g/L) of sucrose. Its dry cell weight increased drastically to 2.3 ± 0 g/L and 3.25 ± 0.07 g/L, respectively. Besides, the P(3HB) content showed drastic increase from 11.5 ± 0.7% to 37.5 ± 0.07%, respectively. The dry cell weight and P(3HB) generated from 1.5, 2.0 and 2.5% (w/v) of sucrose showed similar measurement. The highest P(3HB) concentration obtained was 1.74 ± 0.05 g/L using 25 g/L of sucrose. Although the increasing amount of sucrose should induce more cell growth and PHA accumulation (Page, 1992), the cell biomass and PHB increased with reducing rates possibly due to depletion (limitation) of other essential nutrients that might have been consumed by the bacterial cells. Besides, the consumption and availability of sucrose could be investigated by analysing the residual sucrose in the medium using phenol-sulfuric method (Nielsen, 2010). The excess sucrose might also be fed to the other carbon-consuming characteristic such as extracellular polysaccharide (alginate) production.

CONCLUSION

A. vinelandii mutant Δ*Avin_16040* strain was used to accumulate P(3HB). Three folds higher P(3HB) was accumulated by the mutant strain compared to its wild type strain in the MDA medium. Nine types of mineral media were used for strain cultivation and PHA accumulation including Ashby's, Burk's and DSMZ-Azotobacter, minimal medium PHA and their derivatives which contain or lack calcium carbonate and/or urea (nitrogen source). Addition of urea as nitrogen source increased the PHA accumulation and dry cell weight of *A. vinelandii* mutant grown in AM and its derivative MAM, as well as BM. The highest dry cell weight was obtained from MMPHA medium (3.5 ± 0.1 g/L) while and the highest PHA yields were obtained from MMPHA (1.5 ± 0.03 g/L), AM (1.3 ± 0.28 g/L) and MAM (1.48 ± 0.14). MAM and AM showed the highest PHB accumulations of 71 ± 1 and 50 ± 1% respectively. Further investigations could be carried out by conducting response surface methodology to determine the optimised medium for PHB production, as well as large-scale fermentation and incorporation of other monomers into P(3HB) for development of new applications.

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