Inexpensive fed-batch cultivation for high poly(3-hydroxybutyrate) production by a new isolate of *Bacillus megaterium*

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This research aimed at increasing the cell density and production of the homopolymer polyhydroxybutyrate (PHB) by *Bacillus megaterium* BA-019, using renewable and inexpensive bioresources as a substrate. A higher cell density and a greater PHB production level were obtained by using sugarcane molasses and urea as carbon and nitrogen sources, respectively. The limitation of nitrogen at a C/N molar ratio of 25 resulted in enhanced cell growth and PHB production in batch cultures. Fed-batch cultivation with the feeding nutrient composed of MSM with sugarcane molasses, urea and trace elements, and controlled by a pH-stat feeding control, lead to a significantly enhanced cell concentration and PHB production. The optimal feeding medium in this system required a higher total sugar concentration (400 g/l) and a C/N molar ratio of 10 mol/mol. Under these conditions the highest attained cell mass (72.6 g/l DW) and PHB content (42% of cell dry wt.) were achieved in a short cultivation time (24 h), leading to improved PHB productivity (1.27 g/l/h). However, dissolved oxygen was limiting and thus the system is likely to be suboptimal and capable of even further improvements to the PHB production rate.

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[Key words: Poly(3-hydroxybutyrate) (PHB); *Bacillus megaterium*; Sugarcane molasses; Nitrogen limitation; Fed-batch cultivation]

Poly(3-hydroxybutyric acid) (PHB), an intracellular microbial thermoplastic, is the principal polyhydroxyalkanoate (PHA) that is widely produced by many bacteria including *Bacillus megaterium*. This biologically produced polyester has received much attention since it is biodegradable, whilst various kinds of renewable resources can be used as substrates for its synthesis by the producing bacterial strains (1), making it adaptable to local or regionally available supplies rather than having to import specific products.

Regardless of production methodology, PHB has attracted much commercial interest as a biodegradable plastic material because its physical properties are remarkably similar to those of the non-renewable petroleum based polypropylene. It is biocompatible, nontoxic and has a low immunogenicity and is suitable for medical applications. At present, PHB and its copolymers and terpolymers can be used as bulk commodity plastics, e.g., in agricultural and medical applications, due to their biodegradability and biocompatibility, and future applications are potentially more widespread.

However, despite the apparent benefits of using PHB as a replacement for petrochemical-derived plastics, the use and distribution of PHB is currently limited by its relatively high production cost compared to petroleum based polymers like polypropylene (2, 3). Alternatively, the production cost of PHB by contained natural microbial biotechnology, such as *B. megaterium*, as well as recombinant *E. coli*, could be lowered by enhancing bacterial cells to produce a larger amount of polymer, by using cheaper raw materials as medium components and by combining both approaches together. One approach to attaining a higher PHB yield is thus to promote bacterial growth during fermentation by feeding appropriate carbon and nitrogen sources at a suitable concentration and rate so as to attain a high cell density and PHB production rate.

There are plenty of low-cost, renewable, carbon-containing raw materials available around the world. Sugarcane molasses is one such renewable agricultural commodity. Besides simple carbohydrates as carbon sources, sugarcane molasses contains vitamins and other minor constituents that can be used as sources of carbon and as growth factors (4).

In general, the optimization of fermentation conditions has proved to be successful in substantially enhancing the product yield and productivity of many bioprocesses (5). Given that PHB synthesis is known to be favored by environmental stresses such as nitrogen, phosphate or oxygen limitation (6), this is an obvious starting point. For the optimal growth and production of PHB by *Alcaligenes eutrophus* and *Halomonas boliviensis*, (NH4)2SO4 and amino acids have been used as the principal nitrogen source (7–10), whilst ammonium phosphate was used as a combined nitrogen and phosphorus source in the continuous cultivation of *Serratia* sp. in an air-lift fermenter for biofilm production (11, 12). However, these are all relatively expensive nitrogen sources at commercial levels. The use of monosodium glutamate as a nitrogen source at a concentration of 1% (w/v) for PHB production by *Pseudomonas fluorescens* A2a5 (13) is of potential commercial application but of less general global application, and is also of equivocal cost savings. In contrast, urea, which is a low cost and readily available nitrogenous compound, was reported to...
To investigate the effects of pH and dissolved oxygen levels upon PHB production in batch cultivation, the following conditions were studied: 

- pH: 6.0, 7.0, or 8.0 (compared to an uncontrolled pH condition)
- Dissolved oxygen concentration held at 40, 60, or 80% relative air saturation as indicated.

The results revealed the importance of the studied parameters on the bacterial growth and PHB yield attained.

### MATERIALS AND METHODS

#### Bacterial strain

*B. megaterium* BA-019, which was isolated from soil in Thailand, and identified in our laboratory (16S rDNA sequence similarity, morphological and biochemical characteristics; unpublished data in Kamoljarasopa, K. 2004 M.Sc. thesis) was used in this study. The stock culture was maintained on nutrient agar slants overlaid with 20% (v/v) glycerol and kept at -20 °C.

#### Culture media and inoculum preparation

Basal culture medium (BCM) was composed of 10 g/l yeast extract, 10 g/l polypeptone, 5 g/l beef extract, 5 g/l NaCl and 10 g/l sucrose. For PHB production a complex medium composed of 2.0 g/l KH2PO4, 0.6 g/l Na2HPO4, 1.0 g/l MgSO4.7H2O, 0.1 g/l yeast extract and 1 ml/l of trace element (1.3 g/l ZnSO4.7H2O, 20.0 g/l CaCl2, 0.2 g/l FeSO4.7H2O, 0.6 g/l (NH4)6Mo7O24.4H2O and 0.6 g/l H3BO3), was used. Sucrose and cane molasses were used as carbon sources at the indicated concentrations, whilst (NH4)2SO4 and urea were likewise used as nitrogen sources. The carbon sources were sterilized separately at 110 °C for 10 min and then aseptically added into the flask containing the other components at room temperature. The pH of the final culture medium was adjusted to 7.0 before bacterial inoculation.

A starter (seed) *B. megaterium* inoculum was prepared by incubating a single *B. megaterium* colony in a conical flask with 50 ml of BCM at 30 °C with shaking at 200 rpm for 6 h.

#### Shake flask culture

The effect of different carbon sources on bacterial growth and PHB production

PHB production was carried out using MSM media supplemented with either sucrose or molasses at 20 g/l as a carbon source with or without the addition of 0.8 g/l ammonium sulphate or urea as a nitrogen source, as indicated in the text, to form production media. Two ml of the seed *B. megaterium* inoculum was added into 50 ml of production medium and incubated at 200 rpm, 30 °C for 36 h. Samples were then taken for analysis.

#### Comparison of bacterial growth and PHB production with different nitrogen sources

To investigate the effect of the nitrogen source on the growth and PHB production by *B. megaterium*, MSM medium supplemented with cane molasses (20 g/l) as a carbon source was further supplemented with either urea or ammonium sulphate at 0.8 g/l as the investigated nitrogen sources. After seeding with 2 ml of *B. megaterium* inoculum in 50 ml of media, cultures were grown at 30 °C with shaking at 200 rpm for 36 h. Samples were then analyzed at various time intervals for further analysis.

#### Fermentation studies

The effect of different C/N molar ratios on PHB production in the batch cultivation of *B. megaterium*

The effect of the molar ratios of PHB production was investigated compared with that of authentic PHB. The melting temperature (Tm) and glass transition temperature (Tg) of PHB produced by *B. megaterium* BA-019 were determined by DSC (Differential scanning calorimetry), and the molecular weight was estimated by GPC (gel permeation chromatography) following the method described by Mergaert et al. (17).

Dry cell weight

The culture broth was centrifuged (Kubota 6500 centrifuge) at 10,000 rpm for 10 min, and the supernatant was analyzed for residual sugar and nitrogen content. The harvested cells (pellet) were washed twice with distilled water by resuspension and centrifugation as above, and then transferred to a preweighed aluminum dish. The cells were dried at 80 °C until a constant weight.

PHB concentration

Intracellular PHB was determined by the method developed by Comeau et al. (16) using a gas chromatograph (Varian, CP-3800 GC) equipped with a Carbowax PEG capillary column and flame ionization detector (FID). Benzoic acid was used as an internal standard.

 Nitrogen concentration

Ammonium sulphate concentration was determined by the method of Kemper (18). Urea concentration was determined using the method of Jutter et al. (15). The purified PHB produced by *B. megaterium* BA-019 was purified by the method of Jutter et al. (15). The purified sample was analyzed by GC (16) in direct comparison with authentic PHB. Chemical characterization was performed by IR, 1H-NMR and 13C-NMR and the elemental analysis of C and H was investigated compared with that of authentic PHB. The melting temperature (Tm), and glass transition temperature (Tg) of PHB produced by *B. megaterium* BA-019 were determined by DSC (Differential scanning calorimetry), and the molecular weight was estimated by GPC (gel permeation chromatography) following the method described by Mergaert et al. (17).

Statistical analysis

Data are presented as the mean derived from three independent repeats and were analyzed by ANOVA, using p < 0.01 as the level of significant difference.

### RESULTS AND DISCUSSION

The effects of different carbon and nitrogen sources

For *B. megaterium* BA-019, the cell growth, as determined by dry weight biomass (DCW), and the total PHB content, determined as the proportion of the bacterial DCW, were significantly improved when molasses rather than sucrose was used as a carbon source regardless of the nitrogen source (Table 2). Thus, the attained dry cell weight with molasses as a carbon source was 2.5 to 2.6 fold higher than that for sucrose with (NH4)2SO4 and urea as a nitrogen source, respectively, whilst the corresponding increase in PHB levels was slightly lower at 1.75 to 1.8 fold, respectively. With respect to the nitrogen source, urea was found to be a better nitrogen source than (NH4)2SO4 regardless of the two carbon sources, for both cell growth (biomass) and the PHB content of the cells. Thus, the highest cell biomass and PHB content were attained using molasses and urea as carbon and nitrogen sources, respectively. This is encouraging, since these sources provide a good nitrogen source that supports both the growth of cells and PHA biosynthesis (14).

This work reports on a study which aimed to determine the suitable conditions (e.g. carbon and nitrogen sources, C/N ratio, limitation of nitrogen, DO, pH and feeding strategies) under a fed batch culture system that would promote optimal bacterial growth and PHB production. The results revealed the important effects of the studied parameters on the bacterial growth and PHB yield attained.

### TABLE 1. Composition of the feeding nutrients used in the fed-batch cultivation of *B. megaterium* BA-019

<table>
<thead>
<tr>
<th>Condition</th>
<th>Feeding nutrient</th>
<th>C/N</th>
<th>Total sugar</th>
<th>Urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Molasses</td>
<td>–</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>2</td>
<td>Molasses + urea</td>
<td>25</td>
<td>150</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Molasses + urea + minerals</td>
<td>25</td>
<td>150</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Molasses + urea</td>
<td>25</td>
<td>400</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>Molasses + urea + minerals</td>
<td>10</td>
<td>400</td>
<td>40</td>
</tr>
</tbody>
</table>

### TABLE 2. The effect of different carbon and nitrogen sources on *B. megaterium* BA-019 cell growth and PHB accumulation

<table>
<thead>
<tr>
<th>C-source</th>
<th>N-source</th>
<th>Time (h)</th>
<th>DCW (g/l)</th>
<th>PHB content (g/l)</th>
<th>Productivity (g/l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose Ammonium sulphate</td>
<td>24</td>
<td>196</td>
<td>28.57</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Sucrose Urea</td>
<td>24</td>
<td>2.83</td>
<td>30.20</td>
<td>0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>Molasses Ammonium sulphate</td>
<td>36</td>
<td>6.25</td>
<td>49.92</td>
<td>0.09</td>
<td>0.01</td>
</tr>
<tr>
<td>Molasses Urea</td>
<td>36</td>
<td>7.05</td>
<td>55.46</td>
<td>0.11</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Data are the mean derived from three independent repeats. Means with different superscript letters (a, b) within the same column are significantly different at the p<0.01 level (ANOVA Test, DCW = dry cell weight).
represent the cheaper and more readily available carbon and nitrogen sources. The results attained here for molasses are in agreement with other organisms where unpurified carbon sources, such as molasses, malt extract and corn syrup, were reported to enhance PHB production in comparison with purified sugars (glucose, fructose and sucrose) in the Azotobacter vinelandii strain UWD (20), whilst PHB production by A. eutrophus was stimulated when molasses was added to the culture medium (4). This effect might be caused by other nutrients in molasses (besides glucose, fructose and sucrose), including organic acids, minerals, and vitamins such as thiamine, riboflavin and pyridoxine. These function as growth factors, and thus may result in a higher cell growth and PHB production. Note that the same % (w/v) of urea and ammonium sulphate will provide essentially the same concentration of available nitrogen (one mole per 60.6 or 61 g, respectively). That urea was found to serve as a better nitrogen source may be due to the ability of *B. megaterium* BA-019 to assimilate urea better than the other nitrogen compounds. Urea is a small, uncharged polar molecule and, in contrast to (NH₄)₂SO₄, which exists in ionic form, the uptake rate of urea across the cell membrane is less pH dependent and faster than that of (NH₄)₂SO₄ (21). Certainly, the use of a cheap nitrogen source like urea can reduce the PHB production cost if the bacterial strain used can efficiently utilize it (21, 22). Urea is one of the most inexpensive nitrogen sources, some 3.6 fold cheaper than (NH₄)₂SO₄.

Given the most likely method to reduce the production cost of PHB is to use inexpensive medium compositions, *B. megaterium* BA-019 is thus a promising strain as a PHB-producer, being able to efficiently grow and produce PHB on cheap carbon (molasses) and nitrogen (urea) sources.

### The effect of varying the C/N molar ratio upon cell growth and PHB accumulation

The effect of the C/N molar ratio, using a fixed molasses concentration of 20 g/l and varying the urea concentration from 0.2 to 2 g/l, upon *B. megaterium* BA-019 cell growth (dry weight biomass; DCW) and PHB accumulation (% of bacterial DCW) is summarized in Table 3. Both the cell biomass and PHB accumulation were significantly greater at a C/N ratio of 25 than ratios either higher or lower than this, with for example an over two fold greater PHB productivity noted at a C/N ratio of 25 than at 10 or 50. The cultivation time in the jar fermentor was remarkably shortened, some three fold more productivity noted at a C/N ratio of 25 than at 10 or 50.

<table>
<thead>
<tr>
<th>C/N (mol/mol)</th>
<th>DCW (g/l)</th>
<th>PHB conc. (g/l)</th>
<th>PHB content (% DCW)</th>
<th>Yp/s (g PHB/g sugar)</th>
<th>Productivity (g PHB/g/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>6.72</td>
<td>2.95</td>
<td>29.00</td>
<td>0.108</td>
<td>0.16</td>
</tr>
<tr>
<td>25</td>
<td>8.24</td>
<td>4.16</td>
<td>50.50</td>
<td>0.268</td>
<td>0.35</td>
</tr>
<tr>
<td>50</td>
<td>6.04</td>
<td>1.83</td>
<td>30.10</td>
<td>0.222</td>
<td>0.15</td>
</tr>
<tr>
<td>100</td>
<td>3.63</td>
<td>0.98</td>
<td>27.00</td>
<td>0.155</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Data are the mean derived from three independent repeats. Means with different superscript letters (a – f) within the same column are significantly different at the p≤0.01 level (ANOVA Test). DCW = dry cell weight.

### The effects of pH and dissolved oxygen concentration upon cell growth and PHB accumulation

The influence of media pH and dissolved oxygen levels on the *B. megaterium* BA-019 biomass yield and PHB accumulation attained is summarized in Table 4. Considering dissolved oxygen (DO), which was only varied across media maintained at pH 7.0, the highest cell biomass and PHB content, Yp/s and productivity were all attained at 60% DO, although this was not statistically significantly higher than that attained at 80% DO. Considering the media pH, the highest PHB content, Yp/s and productivity were all obtained when the media pH was maintained at 7.0. In contrast, the cell biomass was greatest at pH 8.0 and in media without pH control. This might be due to the fact that pH 7.0 is the optimal value of the key enzymes in the PHB biosynthetic pathway. Therefore, at higher or lower pH than the optimal, enzyme activity decreases. The reason for this observed optimal pH of 8.0 is not clear from the data of this study, and may simply reflect the imposed costs of maintaining internal homeostasis. Nevertheless, these results are consistent with Grothe et al. (25), who reported that pH changes reduced culture performance and surmised that the initial media pH value may affect the availability of required trace elements in the culture medium. Within pH 7.0 controlled media, a DO level of 60% air...
saturation appeared to be the optimal DO concentration for growth and PHB synthesis. At a DO level of 40% the cultivation time was prolonged; hence, productivity was decreased dramatically. This might be caused by insufficient oxygen in the culture broth to meet aerobic anabolic demands. However, higher DO levels (80%) significantly reduced the bacterial growth and reduced, but not significantly, the PHB yield attained. This may be due to oxidative and shear stresses that resulted from the high agitation speeds. Thus, future work may benefit from maintaining a constant agitation speed and adjusting the oxygen levels by the addition of enriched air or pure oxygen to evaluate their requirement and benefit first, given the cost to include aerobic anabolic demands. However, higher DO levels (80%) significantly increase the initial outlay and thereafter the daily operating costs. In addition, for repeated batch systems it would significantly increase the operation cost and the cost effectiveness of PHB production. For example, in the case of citric acid production, a continuous production system with recirculation of the fermentation broth after product recovery was developed as a four stage process (28). In this system, a membrane module in the reactor is used to separate the biomass from the effluent, the latter consisting of the fermentation broth with the product. In addition, a repeated fed-batch system to remove some of the culture broth, which contains the carbon source, has been reported for the production of xyitol by recombinant S. cerevisiae (29). The other possible procedure may be the application of a suitable membrane filter, such as the hollow fiber microfilter used to remove mannitol in Lactobacillus intermedius NRRL B-3693 fed-batch and continuous cell-recycle fermentation (30). However, such operations to remove and recover the carbon source in fermentation broth can significantly increase the initial outlay and thereafter the daily operating costs. In addition, for repeated batch systems it would also increase the cultivation time. From an economical point of view, consideration must be taken between the operation cost and the cost effectiveness.

The effect of using a high cane molasses (equivalent to 400 g/l of total sugar) and balanced urea (C/N molar ratio of 25) and minerals In order to obtain higher cell mass and PHB concentration, the sugar cane molasses concentration was increased from 150 g/l to 400 g/l. The results, summarized in Fig. 1, suggest that this feeding nutrient composition was suitable for both the growth of B. megaterium BA-019 and for increased PHB production. The total sugar level was increased after the nutrient was fed for 8 h but then decreased gradually. Initially, an almost tenfold higher cell density (58.93 g/l) and PHB production rate (0.93 g/l/h) was attained after 27 h of cultivation. However, the high cell mass in the culture broth lead to a rapid decrease in the DO which was exhausted at 12 h. The amount of urea also decreased reaching zero after 6 h. It seems likely that nitrogen starvation then caused sugar to rapidly accumulate in the medium (to above 50 g/l) resulting in cell-growth inhibition, cell lysis and excess foaming.

The effect of using a high cane molasses (equivalent to 400 g/l of total sugar) and urea (C/N molar ratio of 10) feeding solution with minerals Given the rapid exhaustion of urea and thus nitrogen limitation in the above high molasses feed solution, in order to try to improve PHB production a feeding solution containing a higher urea concentration (C/N of 10) was investigated. The same pH-stat feeding control beginning at 8 h of cultivation was used and the data is summarized in Fig. 2. The amount of total sugar and urea remained almost constant during the 30 h cultivation. Remarkably, a significant increase in the cell mass of B. megaterium BA-019 was achieved (72.6 g/l), some 1.23 fold greater than that attained before with a C/N ratio of 25 (Fig. 1). Correspondingly a greater PHB production was found (30.5 g/l) at 24 h of cultivation resulting in a shorter cultivation time and higher PHB productivity (1.27 g/l/h). However, presumably due to the larger amount of cells obtained in this cultivation, the DO rapidly decreased to 0% and so would be limiting, and likely to have caused the observed growth inhibition after 24 h. A future challenging study would be to supply a sufficient amount of DO, perhaps by using enriched air as mentioned earlier, for higher cell density and cellular PHB content, but with a shorter cultivation time.

In our work reported here, at the final stage of fermentation, a rather high amount of sugar remained. This may be recyclable through the operation of a continuous cultivation system to further improve the efficiency and cost effectiveness of PHB production. For example, in the case of citric acid production, a continuous production system with recirculation of the fermentation broth after product recovery was developed as a four stage process (28). In this system, a membrane module in the reactor is used to separate the biomass from the effluent, the latter consisting of the fermentation broth with the product. In addition, a repeated fed-batch system to remove some of the culture broth, which contains the carbon source, has been reported for the production of xyitol by recombinant S. cerevisiae (29). The other possible procedure may be the application of a suitable membrane filter, such as the hollow fiber microfilter used to remove mannitol in Lactobacillus intermedius NRRL B-3693 fed-batch and continuous cell-recycle fermentation (30). However, such operations to remove and recover the carbon source in fermentation broths can significantly increase the initial outlay and thereafter the daily operating costs. In addition, for repeated batch systems it would also increase the cultivation time. From an economical point of view, consideration must be taken between the operation cost and the cost effectiveness.
of final product. In the case of larger scale fermentations, such approaches might be suitable since the unit price of products tends to decrease as production scales increase (31), and this is another option for further optimization of the efficiency of PHB production at commercial scales.

A comparison between batch and fed-batch cultures with different feeding strategies In overall summary, a comparison of the total cell biomass and PHB production parameters attained with batch culture of *B. megaterium* BA-019 with a C/N ratio of 25 was compared to that derived from fed-batch cultures with three different nutrient-feeding compositions (Table 5). Fed-batch cultures with molasses, urea and mineral salts exhibited a higher cell mass and PHB concentration, content and productivity. Within this category of nutrient feed, that which contained 400 g/l of total sugar at a C/N ratio of 10 clearly supported the highest cell growth and PHB production and attained, after 24 h of cultivation, a maximal PHB concentration of 30.5 g/l, which is equivalent to 42.1% DCW. Overall, the data summarized in Table 5 suggests that *B. megaterium* BA-019 requires carbon and nitrogen sources as well as minerals for optimal growth and PHB production, but that this can be attained with the cheaper molasses and urea.

Thus, a higher cell mass of *B. megaterium* BA-019 and a greater PHB yield was obtained in the culture medium containing sugarcane molasses as a carbon source, compared to that of refined cane sugar, whilst urea was a more suitable nitrogen source than ammonium sulfate. With a controlled pH at 7.0, DO at 60% of air saturation, trace elements and a high sugar (400 g/l total sugar) level with a C/N molar ratio of 10, the pH-stat fed-batch cultivation of *B. megaterium* BA-019 resulted in a significant increase in cell density and PHB yield making this system and bacterial isolate a promising alternative candidate for PHB bioproduction.

Finally, we compared the results of this study to other studies on PHB production by *B. megaterium* BA-019 using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources, respectively (32, 33), as well as in general. We conclude that although the PHB content in the two *B. megaterium* studies (this and (32)) are nearly equal, a much higher cell mass and PHB productivity is attained in the system reported here. More generally, comparing our result with other studies on PHB production by other bacteria and carbon sources (34–40) in Table 6, we can conclude that the PHB content, DCW and PHB productivity obtained in this study are higher than in several other systems including the transgenic *E. coli* (33); Jo, S. et al. (2007) reported the highest P(3HB) content of 52.5 wt.% in *Corynebacterium glutamicum* was obtained by combining the genetic dosage of phaA and B with codon optimization (41). However, there are other reported systems that can produce a higher PHB content, PHB concentration and productivity. Most notably is that of PHB production by *A. latus* with sucrose as the carbon source (36,37). P (3HB) production by the wild type and recombinant bacterial strains using different substrates was summarized by Tokiwa, Y. and Ugwu, C.U.(2007) (42). However, PHB production in these systems uses more expensive carbon and nitrogen substrates and so overall the economic advantage of these systems over the one reported here is less clear. Moreover, they use human food utilized carbon sources rather than waste products, and thus are potentially more prone to supply competition and unstable prices. In contrast, PHB production in this system, although not optimal (and so has potential for further improvement) is relatively high both in cell mass and PHB content, and requires a short cultivation time. Coupled with the utilization of cheap waste raw materials as carbon and nitrogen sources this is an attractive potential system for PHB production. Certainly, we conclude that our initial aim, to establish a potentially inexpensive PHB production system has been achieved.

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