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RESEARCH PAPER

The Production of Polyhydroxyalkanoate by *Bacillus licheniformis* Using Sequential Mutagenesis and Optimization

Kanokphorn Sangkharak and Poonsuk Prasertsan

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Abstract The purpose of this study was to enhance the production of polyhydroxyalkanoate (PHA) by sequential mutation of *Bacillus licheniformis* PHAs-007, using UV and N-methyl-N'-nitro-N-nitrosoguanidine (NTG). In addition, the effect of nutrient additions and environmental conditions were optimized to increase the production of PHA. *Bacillus licheniformis* PHAs-007 produced high amounts of PHA (64.09 ~ 68.80% of DCW) under both synthetic and renewable substrates. After mutagenesis treatment, mutant M2-12 was selected from 380 strains, based on its high biomass and PHA concentration. The mutant M2-12 gave the highest value of specific growth rate (0.09/h), biomass (22.24 g/L) and PHA content (19.55 g/L) under optimal conditions, consisting of 3% palm oil mill effluent, with no additional trace elements, at 45°C and pH 7. The mutant strain showed higher resistance to substrate concentrations, as well as pH and temperature, than the wild type. The accumulation of PHA was increased by 3.18- fold compared to the wild type, and the production of PHA by the mutant M2-12 was constantly retained over 12 times of cultivation. The mutation and optimization strategy appear to be suitable for producing high density PHA, reducing the medium cost and consequently lowering the production cost. Interestingly, the mutant strain could synthesize the novel PHA copolymers such as 3-hydroxyvalerate and 3-hydroxyhexanoate, which were not produced by the wild type.

Kanokphorn Sangkharak*
COE for Sustainable Energy and Environment, Department of Chemistry, Faculty of Science, Thaksin University, Phatthalung 93110, Thailand
Tel: +66-7460-9634; Fax: +66-7460-9634
E-mail: skanokphorn@yahoo.com

Poonsuk Prasertsan
Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Songkhla 90112, Thailand

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1. Introduction

Plastic garbage has become a major concern in terms of its many negative environmental impacts. A major disadvantage of synthetic plastics is that they do not degrade naturally, and many toxins are produced during their production and combustion [1,2]. Polyhydroxyalkanoate (PHA) is polyester which is naturally produced by bacteria, having similar properties to synthetic plastic, while being completely degraded by PHA depolymerases at a high rate within 3 ~ 9 months. The major drawback of PHA is their high costs [3]. Therefore, the commercial use of PHA is dependent on low production costs and the feasibility of mass production. Owing to their inherent metabolic control systems, micro-organisms usually produce PHA in very low concentrations, and although the yield may be increased by optimizing the cultural conditions, productivity is controlled ultimately by the organism's genome [4]. Consequently, genetic alteration is an attractive route for the process development of this biotechnology. The improvement of microbial strains for the production of PHA has attracted attention in the commercial fermentation process [5]. Many groups have reported their attempts to increase the yield of PHA production by genetic improvements, using mutagenesis *via* mutagenic agents. The use of different mutagenic agents, such as ultraviolet (UV), X-rays and gamma radiation, as well as chemical mutagen including ethyl methane sulfonate (EMS), NTG and mustards, were demonstrated [5-9]. The effect of gamma irradiation on PHA production by *Bacillus flexus* was evaluated by Divyashree and Shamala [32]. Irradiation resulted in cell damage, and aided in the

isolation of 45 ~ 54% PHA on biomass. In addition, the molecular weight of the produced PHA was also increased from 1.5×10^5 to 1.9×10^5 after irradiation. Moreover, the production of PHA by *Rhodobacter sphaeroides* U17 and N20 were improved 2.25- and 3.28-fold (compared to wild type), respectively, after being treated with UV and NTG, respectively [10]. However, no information was available for the development of PHA-production by sequential mutation. This study is the first report of the enhanced production of PHA by *Bacillus* species using repeat and sequential mutagenesis.

The PHA-producing bacteria, *Bacillus licheniformis* PHAs-007 was isolated from our previous study [11]. The strain had an attractive ability to produce a high amount of PHA (64.09 ~ 68.80% of DCW) under both synthetic and renewable substrates. Thus, *B. licheniformis* PHAs-007 was selected for strain improvement throughout this study. The purpose of this study was to improve the production of PHA by mutation. In this study UV and NTG were sequentially used to mutate the bacterial strain, and the effect of nutrients and environmental conditions were optimized for the increased production of PHA.

2. Materials and Methods

2.1. Microorganism and culture media

A wild type bacterium, *Bacillus licheniformis* PHAs-007, identified by Sangkharak and Prasertsan [11], had been subjected to sequential mutagenesis by UV and NTG. The strain was maintained at 37ºC in PHA producing medium ($(NH_4)_2SO_4$ 2 g/L, KH_2PO_4 13.3 g/L, $MgSO_4.7H_2O$ 1.2 g/L, citric acid 1.7 g/L, trace element solution 10 mL/L). The medium was adjusted to pH 7.0 using 5 mol/L NaOH.

Isolation of mutants was achieved through streaking on fresh PHA detection agar plates containing glucose 20 g/L, $(NH_4)_2SO_4$ 2 g/L, KH_2PO_4 13.3 g/L, $MgSO_4.7H_2O$ 1.2 g/L, citric acid 1.7 g/L, trace elements solution 10 mL/L and agar 15 g/L containing Nile blue strain (0.5 mg/mL) [12-14].

2.2. Sequential and repeated mutagenesis

The wild type cells, grown in PHA medium for 24 h, were harvested at the logarithmic phase by centrifugation (10,000 *x g*, 20 min) at 4ºC. After the cell concentration was determined by counting, cells were spread on PHA detection agar. The plates were placed under an ultraviolet lamp (Sylvania G30W) at a distance of 55 cm for various periods of time, (15 sec ~ 30 min) to give a survival ratio of cells of 0.01 ~ 0.1% [5,7]. Following irradiation, the plates were kept in the dark for 1 h and the strains showing a halozone were picked up and selected for NTG treatment.

Selected mutants obtained after UV irradiation were further mutated by NTG. 1 mL of NTG solution (100 mg/mL, 0.2 M pH 6.0 phosphate buffer) was added to 1 mL cell suspension of the strain (5 ~ 8 × 10^8 cell/mL). After incubation at 37ºC on a rotary shaker at 120 rpm, and for different time intervals between 15 and 60 min, the mixture was diluted 1,000 times with sterile buffer, and 1 mL suspension was spread on to the PHA medium to calculate the percentage survival. Samples with a death rate of ca. 90% were subjected to subsequence isolation. The above treated suspension (100 μL) was spread on different screening plates. The plates were incubated at 37ºC for 2 days and the mutants were selected on the basis of clearance zones in detection media [7]. The mutant strain yielding the highest levels of PHA was subjected to repeat treatment by UV and NTG. After treatment, mutant strains were screen and tested for biomass and PHA production, and the mutant yield the highest level was selected for the optimization study.

2.3. Effect of nutrient and environmental conditions

Optimization studies were conducted the conventional method of changing one factor at a time. Starter cultures were prepared by inoculating the selected strain in 100 mL PHA medium and incubating them on a shaker (150 rpm) at 37ºC for 48 h. The starter culture containing 1.5×10^4 viable cells/mL was inoculated (10%) to medium supplemented with various nutrient compositions, and varying carbon sources and concentrations (glucose, municipal wastewater, palm oil mill effluent (POME), glycerol, and molasses at 0 ~ 10%) and trace elements within the solution (with or without). Furthermore, the effect of environmental conditions, including initial pH (4 ~ 10) and incubation temperature (25 ~ 45ºC) were also investigated. Growth (in term of dried cell weight, DCW), PHA content and PHA composition were determined at the end of cultivation (60 h).

2.4. Time course on PHA production

The time course of PHA production from the selected strain under the optimal condition was studied. The experiments were conducted in a 5-L glass fermentor, containing three six bladed Rushton turbine impellers (40 mm dia.), and was pH controlled. Dissolved oxygen (DO) foam and temperature probes were connected to the controller with an agitation speed of 150 rpm. Samples were taken at various time intervals to determine growth (DCW) and PHA concentration during 60 h cultivation. The kinetic parameters, including specific growth rate (μ), production rate (Pm), conversion yield of product to cells ($Y_{p/x}$) and maximum productivity (R_m) were evaluated following the methods of Prasertsan *et al.* [15] and Gopinath *et al.* [16].

2.5. Analytical methods

The bacterial culture was harvested at the end of fermentation and growth was monitored by measuring the absorbance at 660 nm, followed by the method described by Shimizu *et al.* [17]. For qualitative determination, PHA was analyzed in whole-cell samples or after extraction with chloroform and purification by repeated precipitation from a chloroform solution with ethanol. The PHA content and composition were determined by gas chromatography-mass spectrometry (GC-MS), and benzoic acid was used as the internal standard [18-20]. PHA content is defined as the percentage of DCW, *i.e.,* 100 x (g PHA / g DCW). The polymer structure was confirmed by ^{1}H nuclear magnetic resonance spectra (NMR) (Bruker DPX-300). The spectra were recorded at 150.91 and 600.17 MHz using $CHCl_3$ as solvent. For each analysis, 40 mg of sample and 4 mL of solvent were employed [35].

The thermal properties and PHA purity were determined using a differential scanning calorimeter (DSC). Approximately 10 mg of sample was used for each analysis. The samples were heated from -100 to 250°C at a rate of 10°C/min. The first and second cooling runs were carried out at rates of -100 and -10°C/min, respectively. From the first and second heating runs, glass transition temperature (T_g), melting temperature (T_m), crystallization temperature (T_c) were obtained. The heat of crystallizing fusion (X_c) was determined from the heat of melting fusion (ΔH_m) and the melting enthalpy of 100% crystalline PHB ($\Delta H^0{}_m$ = 146 J/g) [36]. In additional, Molecular weight was estimated by gel permeation chromatography (GPC), as previously described by Li *et al.* [35] with polystyrene and $CHCl_3$ used as a standard and solvent, respectively. All data were calculated as the mean and standard derivation of three parallel studies.

3. Results and Discussion

3.1. Sequential mutagenesis and screening for the production of PHA

A local isolate *Bacillus licheniformis* PHAs-007 identified by Sangkharak and Prasertsan [11], had been selected and subjected to successive mutagenic treatments using UV irradiation and NTG. The strain PHAs-007, isolated from pickle waste, produced a high amount of PHA (64.09 ~ 68.80% of DCW) when grown on both synthetic and renewable substrates. The production of PHA after sequential mutagenesis by various mutants is indicated in Table 1. *B. licheniformis* PHAs-007 was initially exposed to UV irradiation. The best UV-mutant strain, M1-34, produced 6.88 g PHA/L. However, PHA production was only slightly improved upon from that of the wild type (6.58 g/L). UV light may cause two adjacent pyrimidine residues (cytosine or thymine) to form a dimer. They may cause error at the subsequent DNA replication resulting in mutation. The gene responsible for the production of PHA may increase in the DNA of bacteria due to the mutation, increasing PHA produrtion [21]. The UV treated mutant strain was further treated with NTG, and one of the resulting mutants, M2-12, had the highest production of PHA, showing a 139.15% production improvement (1.68 fold) compared to the wild type. On the other hand, the production of PHA was significantly decreased from 5.15 to 4.05 g/L after repeated treatments with UV and NTG, respectively.

The results indicated that sequential mutagenesis was appropriate for improving PHA production. The PHA concentration was increased by 139.15% compared to the wild type after the first round of UV and NTG. However, repeated treatment was not suitable for increasing the production of PHA, but resulted in decreases (79.78 ~

Table 1. The production of polyhydroxyalkanoates by the mutant *B. licheniformis* produced through various mutagenic treatments

Mutagenic treatment	Strain	Biomass (g/L)	PHA production (g/L)	Production improved [a](%)
None	PHAs-007 (wild type)	10.45 ± 0.12	6.58 ± 0.10 (62.97% of DCW[b])	100.00
1st round mutagenesis				
UV[c] irradition	M1-34	10.66 ± 0.54	6.88 ± 0.12 (64.54% of DCW)	102.45
NTG[d]	M2-12	12.04 ± 0.22	10.55 ± 0.32 (87.62% of DCW)	139.15
2nd round mutagenesis				
UV irradiation	M3-6	10.25 ± 0.21	5.15 ± 0.10 (50.24% of DCW)	79.78
NTG	M4-10	10.07 ± 0.12	4.05 ± 0.20 (40.22% of DCW)	63.87

[a]Percentage of PHA production compared with the wild type strain.
[b]% of DCW, 100 x (g PHA / g DCW); [c]UV, ultraviolet irradiation; [d]NTG, N-methyl-N'-nitro-N-nitrosoguanidine.

61.55%) after a second round of mutagenesis. As this mutant was again treated, no further improvement was observed. This may be due to the application of a mutagenic agent to the wild type strain initialy, followed by a plateau effect of the enhanced characteristic. No further improvement was found after repeated treatment with UV/NTG [21]. The above result suggested that mutations are considered to have the potential to change bacterial PHA content. Similar results were reported by Katircioglu *et al.* [22] for the hyper-production of PHA. Acriflavin, 5-bromourasil (80 ~ 180 μg/mL) and UV light (254 nm) were used as chemical and physical mutagen. 8 of 59 mutant strains of *Bacillus* sp. gave the yield of PHA in the range of 17.37 ~ 63.45% of DCW, which were higher than the parent strains (15 ~ 48% of DCW). The production of PHA by *Rhodobacter sphaeroides* U17 and N20 were improved 2.25 and 3.28-fold (compared to the wild type) after a single treatment of both UV and NTG, respectively [2,10].

3.2. The stability of the mutant *B. licheniformis* strain M2-12

The stability of mutant *B. licheniformis* M2-12 for biomass and PHA production was determined by successive subculturing on PHA production agar for over one year. The mutant was subcultured every month and evaluated for its ability to stably produce biomass and PHA. The mutant M2-12 maintained the same production yield after being subcultured 12 times, indicating that the mutant has heritable character. Mutant *B. licheniformis* M2-12 was found to continuously produce high volumes of biomass, of a high PHA concentration, when grown on PHA producing medium.

3.3. Effect of nutrient and environmental conditions

Effects of carbon sources and concentrations on PHA production were studied from the mutant *B. licheniformis* strain M2-12 in PHA producing medium, where carbon sources, including glucose, municipal wastewater, POME, molasses and glycerol were supplemented at 0 ~ 10%. The production of biomass and PHA by the mutant strain was dependant on the substrates used. However, the biomass and PHA concentration increased with increasing carbon concentration. The highest overall PHA production levels were 18.31 ± 2.51 and 16.23 ± 1.02 g/L, respectively, from a substrate of 3% POME followed by medium supplemented with 4% glucose, yielding 12.04 ± 1.23 g biomass/L and 7.35 ± 1.59 g PHA/L (Table 2). A slight decrease in production was observed when concentration of substrates more than 4% (w/v). GC-MS was used to determine the composition of PHA from the mutant strain. Only PHB was accumulated in medium containing glucose, municipal waste, molasses and glycerol as sole carbon source. Peak of 3-hydroxybutyric acid was observed at the retention time of 5.63 min. However, the co-production of copolymer, which was not produced by the wild type (such as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-co-hydroxyhexanoate) [P(3HB-*co*-3HV-*co*-3HH)]) was also observed by mutant M2-12 in the range of 88.64% of DCW with HV and HH unit fractions of 12 and 8 mol% when cultivated in POME (Table 2). The gas chromatograms for PHA from POME, corresponding to propyl ester of 3-hydroxybutyric acid, 3-hydroxyhexanoic acid and 3-hydroxyvaleric acid, were detected at retention times of 5.63, 7.49, and 8.12 min, respectively, for PHA from mutant *B. licheniformis* strain M2-12. The retention time of individual peaks of methyl ester was matched with that of 1:1:1 mixture of HB, HV and HH copolymer. The thermal behavior of the samples was investigated by DSC. The thermal properties were determined for the purified samples, and those for commercial PHB are shown in Table 3. There was good agreement among data from various repeat samples. The thermogram of PHA samples run by the DSC showed only a single sharp peak melting temperature. The data suggested that the isolated PHA from *B. licheniformis* was of high purity. The result from GC-MS and thermal properties of copolymer indicated the formation of P(HB-*co*-HV-*co*-HH) by the mutant M2-12. The structure of the copolymer was analyzed by ^{1}H NMR. However, the NMR spectrum was overlapped and not easily clarified (data not shown).

P(3HB-*co*-3HV-*co*-3HH), a biodegradable polymer, has been considered for commercial potential because it is more flexible and less brittle than PHB or mcl-PHA, and it also possesses a lower melting point [23]. The organic composition of raw POME from various plants was already identified by Malaysia Palm Oil Board [37]. Lactic acid and acetic acid are the major components, in the range of 3 ~ 5 g/L. Additionally, formic acid, butyric acid and propionic acid were also present (0.1 ~ 0.3 g/L). Therefore, the mixed compositions of PHA were expected to be accumulated. Interestingly, the present study indicates the feasibility of production of useful polymers by the mutant strain of *B. licheniformis* under anaerobically treated POME. The biomass and PHA concentration from mutant M2-12 was about 2.94 times more than that produced by the parent strain *B. licheniformis* PHAs-007 and the PHA content was increased from 63.02 to 88.64% of DCW when POME was utilized as the sole carbon source (Table 2). The mutant was also found to be more tolerant to high concentrations of pure substrate (glucose) and wastes, including POME, municipal wastewater, molasses and glycerol compared to the wild type.

The effects of trace elements on growth and PHA production from *B. licheniformis* M2-12 cultivated in optimized medium (agar supplemented with 3% POME)

Table 2. The production of biomass (DCW) and PHA by mutant *B. licheniformis* strain M2-12 on medium supplemented with various carbon sources

Microorganism	Carbon sources and concentration	Biomass (g/L)	PHA production		Polymer composition (mol%)			Reference
			Concentration (g/L)	Content (% of DCW)	3HB	3HV	3HH	
B. licheniformis PHAs-007 (wild type)	2% Glucose	ND[a]	ND	ND				[11]
	2% municipal wastewater	5.15 ± 0.97	2.11 ± 0.10	40.97	100			
	2% POME	6.22 ± 1.05	3.92 ± 0.41	63.02	91	9		
	2% Molasses	5.54 ± 1.01	2.25 ± 0.57	40.61	100			
	2% Glycerol	1.18 ± 0.24	0.02 ± 0.00	1.69	100			
B. licheniformis M2-12 (mutant)	4% Glucose	12.04 ± 1.23	7.35 ± 1.59	61.05	100			This study
	3% municipal wastewater	9.53 ± 1.44	4.27 ± 1.21	44.81	100			
	3% POME	18.31 ± 2.51	16.23 ± 1.02	88.64	88	12	8	
	3% Molasses	10.00 ± 1.57	4.22 ± 1.11	42.20	100			
	3% Glycerol	5.01 ± 0.09	1.12 ± 0.14	22.36	100			

[a]ND = not determined; 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HH, 3-hydroxyhexanoate.

Table 3. Thermal properties obtained from isolated PHA by mutant *B. licheniformis* strain M2-12 cultivated under optimal medium containing 3% POME compared with the commercial PHA

Sample	Molecular weight (x10^3Da)			T_g (^oC)	T_m (^oC)	T_c (^oC)	ΔH_m (J/g)	ΔH_c (J/g)
	M_w	M_n	M_w/M_n					
Commercial								
PHB[b]	1,635	847	1.93	-3.08	163.30	91.54	70.64	54.56
PHV[b]	1,056	815	1.30	-15.09	112.27	58.37	73.31	51.81
P(HB-*co*-HV)[c]	1,301	694	1.87	-0.80	150.00	64.30	87.60	-
PHH[b]	272	206	1.32	-28.19	-	-	-	-
P(HB-*co*-HV-*co*-HH)[c]	535	384	1.39	-7.87	94.20	-	-	-
Isolated polymer								
PHB	1,775	940	1.89	5.00	173.10	42.90	77.60	53.00
P(HB-*co*-HV-*co*-HH)	970	618	1.57	-1.01	102.77	-	26.92	-

[a]$X_C = \Delta H_m$ / 146 J/g.
[b]Data obtained from [40].
[c]synthetic polymer: P(HB-*co*-HV) 1:1; P(HB-*co*-HV-*co*-HH) 1:1:1.
Molecular weight: M_w = weight average molecular weight, M_n = number-average molecular weight; Thermal properties: T_g = glass transition temperature, T_m = melting temperature, T_c = crystallization temperature, ΔH_m = heat of melting fusion, X_c = heat of crystallizing fusion.

were also studied. It was found that vitamins had the least effect on cellular growth (18.28 ± 2.01 g/L) and had no effect on PHA concentration or content (16.24 ± 1.55 g/L and 88.84% of DCW). Trace elements which often limit the growth of bacteria include K, P, S, Ca, and Fe. Microelements, such as Co, Cu, Zn, Mo, and Mn are also needed for bacterial growth. Metal ions K^+, Mg^{2+}, Fe^{3+}, Ca^{2+}, Mo^{6+}, Mn^{2+}, Co^{2+}, and Zn^{2+} in the medium effect the biosynthesis of PHA [10]. The suitable concentrations of trace elements are required for PHA productivity. Therefore, POME contains substantial quantities of valuable trace elements, including P, K, Mg, Ca, B, Fe, Mn, Cu, and Zn that vary according to the degree of treatment process [38].

From an economic point of view, as the potential for the production of PHA is limited by the availability and costs of the substrate, the cost of carbon source itself accounts for 40 ~ 50% of the total production cost [39]. *B. licheniformis* M2-12 showed the ability to grow and accumulate PHA in the inexpensive medium without any nutrient addition. However, another significant factor for lower production costs is the productivity. Therefore, bioreactor trials and applications of these technologies are currently underway.

The significant effects of pH on PHA accumulation during batch experiments were therefore highlighted. Therefore, the effect of initial pH was investigated by adjusting the pH of the optimal medium between 4 to 10. The highest productivity of biomass (21.34 g/L) and PHA concentration (18.27 g/L) was achieved at pH 7. Under acidic (pH 2 ~ 5) and alkaline conditions (pH 10), either cell growth or PHA production was significantly affected. Like pH, temperature

is also a considerable factor which affects the PHA yield. The highest productivity of biomass and PHA was observed at temperatures ranging from 35 to 50ºC. The highest productivity of biomass (21.54 g/L) and PHA concentration (18.75 g/L) was achieved at 45ºC. On the other hand, the parent strain yielded the maximum biomass (6.22 g/L) and PHA (3.92 g/L) in the medium supplemented with 2% POME at 37ºC and pH 7.0. Therefore, the mutant strain showed higher resistance to substrate concentration, pH and temperature than the wild type. Normally, *B. licheniformis* has an optimal temperature of 37ºC for PHA production. However, mutant strain showed an optimum at 45ºC. Due to the application of mutagenic agent it may cause replication error and change amino-acids that are encoded in a group of proteins that help to stabilize the internal cellular environment. Also, the development of internal cellular stability may reflect a general increase in overall stability, including thermal, pH and substrate. Even so, the production of PHA by mutant strains appears to hold promising application to industry, since mutants are able to growth under various substrate and environmental conditions such as wastewater, POME, molasses and glycerol with no supplementation of trace elements. Similar results were observed by Naveena *et al.* [24] for the production of thrombinase, and by Gopinath *et al.* [25] for the degradation of Congo red by mutant strain of *Streptomyces venezuelae* and *Bacillus* sp., respectively.

3.4. Growth and PHA production kinetic analysis under optimal conditions

The parent and mutant strains were compared for the production of PHA. *B. licheniformis* mutant M2-12 that was cultivated in optimal medium (containing 3% POME) without any supplemented of trace elements and controlled at pH 7 under cultivation temperatures of 45ºC. The cultivation was performed in a 5-L fermentor with an aeration rate of 1.0 vvm and with an agitation speed of 150 rpm. The production of PHA reached a maximum at 48 h of incubation by the parental strain. However, the mutant reached the maximum production of PHA at 36 h, whereas PHA concentration and PHA content showed the same potential as cellular growth. Small amounts of polymer (0.08 ± 0.11 g PHB/L) were first detected at 6 h of growth, and reached a maximum value at the stationary phase, indicating the relationship of *B. licheniformis* PHB production with cell growth. The experiment showed that PHA production was markedly enhanced by sequential mutagenesis and serial optimization. *B. licheniformis* mutant M2-12 was stable and able to produce PHA at high production (88.90% of DCW) so far reported for this bacterium, making this microorganism very interesting for industrial applications (Table 4). At an aeration rate and agitation speed of 1.0 vvm and 150 rpm, respectively, the initial DO was 100% and remained over 65% after 60 h cultivation. The specific growth rate and PHA production rate obtained at optimum

Table 4. Growth and PHA accumulation of *B. licheniformis* strain M2-12 cultivated in optimal medium containing 3% POME without trace element additions, with controlled pH and temperature at 7 and 45ºC, respectively, after 60 h cultivation

Organism	Substrate	PHA % of DCW	References
Bacillus licheniformis			
- wild type	POME	62.97	This study
- mutant M2-12		88.70	
B. cereus M5	Beet molasses	73.80	[26]
B. cereus	Pea shell slurry	41.00	[27]
B. megaterium	Molasses	46.20	[28]
B. megaterium	Beet molasses	52.00	[29]
B. cereus SPV	Glucose	38.00	[30]
Bacillus sp.	Mollasses	7.92	[31]
	Peach pulp	7.78	
B. subtilis EGU163	Glucose	66.60	[13]
B. licheniformis EGU90		15.50	
Bacillus sp. EGU91		2.70	
Bacillus sp. EGU85		5.30	
B. flexus	Sucrose	45.00	[32]
	Sucrose + Castor oil	54.00	
B. cereus	Soluble starch	48.00	[33]
B. sacchari IPT101	Bagasse hydrolysate	62.00	
B. cepacia IPT048	Bagasse hydrolysate	53.00	
B. megaterium	Glucose	59.00	[34]
	Glycerol	60.00	

conditions were found to be 0.09 μ (/h), 0.09 Pm (/h), 0.88 Yp/x and 0.543 Rm (g/L/h) for mutant and 0.05 μ (/h), 0.05 Pm (/h), 0.67 Yp/x and 0.17 Rm (g/L/h) for parental strain, respectively. The kinetic parametric indicated that the maximum productivity (Rm) by *Bacillus licheniformis* mutant M2-12 was 3.18 times greater than the original strain. In addition, the mutant M2-12 also yielded the constant biomass and PHA under large scale production (5-L reactor).

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