openaccess Journal
SCIENCE LETTERS
2013 | Volume 1 | Issue 1 | Pages 5-8

Optimization and effect of Zn^{2+}, Mn^{2+} and Ni^{2+}on poly- -hydroxybutyrate (PHB) synthesis by *Bacillus subtilis* ATCC 6633

Waseem Raza*[1], Wang Yang[2], Muhammad Faheem[1], Sohail Yousaf[3]
[1]College of Resources and Environmental Sciences, Nanjing Agricultural University, 210095, Nanjing, China
[2]Nanjing University of Science and Technology, 210094, Nanjing, China
[3]Department of Environmental Sciences, Quaid-i-Azam University, Islamabad, Pakistan

Abstract
The environmental impact of non-biodegradable polymers shifted the researcher's trend towards the discovery of biodegradable polymers. Among those, poly-β-hydroxybutyrate (PHB) is an attractive polymer that is produced by microorganisms. The aim of the this study was to assess the capability of *Bacillus subtilis* strain ATCC 6633 to accumulate PHB under different growth conditions, in addition, the role of three metal ions (Zn^{2+}, Mn^{2+} and Ni^{2+}) on the synthesis of PHB was also evaluated. The results showed that the maximum PHB accumulation by strain ATCC6633 was obtained after 48 hours of incubation at the temperature of 30°C and at pH 7, while D-mannitol as C source and glycine as N source showed maximum PHB accumulation. The results of the three metal ions effect on PHB accumulation by strain ATCC6633 revealed that the metal ion Ni^{2+} was most effective in increasing PHB accumulation followed by Zn^{2+} and then Mn^{2+}. This research will help to enhance the understanding of the effect of optimum growth conditions and metal ions on PHB accumulation by strain ATCC6633.
Key words: *Bacillus subtilis*, C source, N source, optimization, poly-β-hydroxybutyrate.

Received June 05, 2013; **Revised** August 12, 2013; **Accepted** August 24, 2013
***Corresponding author:** Dr. Waseem Raza; Email: waseem@njau.edu.cn; Phone: +86-15161459593; Fax: +86-2584432420

Introduction

Non-biodegradable polymers are being used in different industries. But the disposal of those polymers wastes is a challenging task and are being thrown off in land mines as garbage and cannot be used for other purposes like biogas production etc. Those polymers affect the percolation of groundwater by reducing the porosity of the land [1]. This is very harmful as groundwater depletion is dangerous. From land, these can be eaten by animals and thus cause animal death because of body choking [2]. Some kinds of polymers release toxins in the environment and thus are the biggest threat to the life of animals and human beings [1]. An attractive alternative to the toxic and non-degradable polymers is the use of biodegradable polymers [3]. But the use of biodegradable polymers is very expensive. Biodegradable polymers were used in the times of the Romans. The first medical use of a biodegradable polymer was Catgut sutures [1]. Applications of biodegradable polymers include sutures, controlled drug release, and tissue engineering. Biodegradable polymers also can be implemented in drug delivery. The polymer slowly degrades into smaller fragments, releasing a natural product, and there is controlled ability to release a drug. The drug slowly releases as polymer degrades. Much interest has been converted to the biosynthesis, physical properties, biodegradation, modification, and utilization of bacterial polyesters because of their potential applications as environmentally friendly materials [1]. These are manufactured via bacterial fermentation and biodegraded to water and carbon dioxide [4]. Among different microbial origin bipoly--mers, poly-(3-hydroxybutyrate) (PHB) are of interest as bio-derived and biodegradable plastics and belongs to the class of poly(hydroxyalkanoates) (PHA) [1]. PHA are thermoplastic polymers whose physical properties range from hard rigid solids to elastomers. Bacterial PHB shows promise, due to its long-term degradation profile and high molecular weight, and it has been available under the trade name Biopol since the early 1980s [1]. PHB is produced by microorganisms (such as *Ralstonia eutrophus* or *Bacillus megaterium*) apparently in response to conditions of physiological stress; mainly conditions in which nutrients are limited [5]. The polymer is primarily a product of carbon assimilation (from glucose or starch) and is employed by microorganisms as a form of energy storage molecule to be metabolized when other common energy sources are not available. Microbial biosynthesis of PHB starts with the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA which is subsequently reduced to hydroxybutyryl-CoA. This latter compound is then used as a monomer to polymerize PHB [6]. PHAs granules are then recovered by disrupting the cells [3]. *Bacillus subtilis* strains are very popular in biotechnology related industries because of their production of diverse variety of metabolites and other products [3, 7]. *B. subtilis* strain ATCC 6633 was reported to produce PHB and it is involved in the sporulation of *Bacillus* spp. [8]. In this report, we optimized the production of PHB by strain ATCC 6633. The optimum incubation time, temperature, pH, C sources and N sources were evaluated. In addition, the effect of three heavy metal ions (Zn^{2+}, Mn^{2+} and Ni^{2+}) on the

synthesis of PHB by strain ATCC 6633 was determined.

Material and Methods

The *Bacillus subtilis* strain ATCC 6633 was provided by School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing China. The strain was grown in tryptic soya agar plates (TSB) and re-cultured after one week.

Determination of PHB

For the determination of PHB, the over night culture of strain ATCC 6633 in nutrient broth was inoculated (1%) in fresh nutrient broth (100 ml) and incubated at 30°C with shaking at 225 rpm for 48h. At the end of this period, the samples were centrifuged for 15 min at 6000 rpm. The cell pellets were washed with sterile and deionized water and dried for 24 h at 60°C. Then the weight of dry cell pellet was determined. Dry cell materials were incubated at 60°C for 1 h with sodium hypochlorite. Supernatant was obtained by centrifugation at 6000 rpm and cell lipids and other molecules (except PHB) were extracted by adding 5 ml ethanol and acetone (1:1 v/v, 96%). PHB was extracted by hot chloroform (adding 10 ml chloroform in water bath). The chloroform was evaporated to obtain crystals of PHB and then the weight of PHB/mg dry cell weight was determined as compared with empty tubes [9].

Optimization experiments

The optimum time for maximum PHB accumulation was determined by preparing liquid culture at described earlier and incubated at 30°C with shaking at 225 rpm. After different intervals, the liquid culture was drawn from flasks and cell dry weighs and PHB synthesis was determined up to three days. The optimum temperature for PHB production by ATCC 6633 was determined by preparing culture medium as described earlier and incubation at different temperatures (20, 25, 30, 35, 40^{o}C) for two days with shaking at 225 rpm. For the evaluation of optimum pH for PHB production by ATCC 6633, the liquid cultures having different pH values (5-9) were prepared and incubated at 30°C with shaking at 225 rpm for 48h. The effect of different C sources and N sources was also evaluated by using 2 g/ml carbon source (glucose, sucrose, D-mannitol, D-fructose, maltose, arabinose) and 0.2 g/ml nitrogen source (proteose peptone, tryptone, yeast extract, L-glycine, L-cysteine, ammonium sulfate and potassium nitrate) amended in minimal salt medium and cell dry weights and PHB accumulation were determined as described earlier.

Metal ion effect on PHB accumulation

The effect of three metal ions (Zn^{2+}, Mn^{2+} and Ni^{2+}) was determined by adding different concentrations of Zn^{2+} as $ZnSO_4$ (100-350 µM), Mn^{2+} as $MnSO_4$ (100-200 µM) and Ni^{2+} as $NiCl_3$ (50-200 µM) in minimal salt medium amended with D-mannitol and glycine as optimum C and N sources and cell dry weights and PHB accumulation were determined as described earlier.

Results and Discussion

There is urgent need to find out new biodegradable polymers that are non-toxic, capable of maintaining good mechanical integrity until degraded and capable of controlled rates of degradation. In addition, there cost of production must be reduced by optimizing their production using cheap sources. Our research was an attempt to optimize and evaluated the effect of three heavy metals on the accumulation of PHB by strain ATCC6633. The results we presented here are interesting and innovative. The results of the incubation time effect on PHB accumulation by strain ATCC6633 showed that maximum PHB accumulation by strain ATCC6633 was obtained after 48 hours and up to 64 hours the accumulation of PHB was remained same. The maximum accumulation of PHB was 7.52% of the cell dry weight which remained around 7.38% until 64 hours (Table 1).

Table 1: Effect of incubation time on the accumulation of PHB by *Bacillus subtilis* ATCC6633.

Time (hrs)	Cell dry weight (g/L)	PHB (mg/L)	PHB (%)
4	0.02±0.002	0	0.02
8	0.06±0.003	0.008±0.0008	0.01
12	0.25±0.02	0.035±0.04	0.01
16	0.35±0.01	16.7±0.57	4.77
20	0.52±0.007	21.7±0.86	4.17
24	0.59±0.01	25.1±0.45	4.25
28	0.66±0.03	30.4±0.58	4.61
32	0.68±0.02	33.7±0.95	4.96
36	0.67±0.01	35.4±0.91	5.28
40	0.66±0.02	40.7±0.79	6.17
44	0.65±0.01	45.7±0.98	7.03
48	0.67±0.004	50.4±1.24	7.52
52	0.67±0.02	49.5±1.21	7.39
56	0.66±0.03	48.7±1.21	7.38
60	0.66±0.01	49.2±0.67	7.45
64	0.66±0.02	50.1±0.58	7.37

The results of optimum temperature and pH study showed that 30°C was optimum temperature while pH of 7 was optimum for maximum accumulation of PHB by strain ATCC6633. Low and high temperatures than 30°C adversely affected the PHB accumulation and it was decreased from 7.7% at 30 °C to 2.5% at 20°C and 2.8% at 40°C. Similarly lower pH was not favorable for PHB accumulation by strain ATCC6633 and PHB accumulation decreased to 1.9% at pH 5 from 7.75% at pH 7. At pH 9, 5.2% PHB accumulation was determined (Table 2).

Table 2: Effect of incubation time, temperature and culture pH on the accumulation of PHB by *Bacillus subtilis* ATCC6633.

Treatments	Cell dry weight (g/L)	PHB (mg/L)	PHB (%)
Temperature			
20 °C	0.25±0.02	6.2±0.8	2.48
25 °C	0.45±0.09	15.8±0.8	3.51
30 °C	0.68±0.05	52.5±0.5	7.72
35 °C	0.57±0.02	29.7±0.6	5.21
40 °C	0.56±0.01	15.8±0.4	2.82
pH			
5	0.35±0.02	6.5±0.4	1.86
6	0.54±0.01	18.7±0.5	3.46
7	0.67±0.01	51.9±0.5	7.75
8	0.61±0.02	32.3±0.8	5.30
9	0.59±0.01	30.4±1.1	5.15

Table 3: Effect different C and N sources on the accumulation of PHB by *Bacillus subtilis* ATCC6633.

Treatments	Cell dry weight (g/L)	PHB (mg/L)	PHB (%)
C sources			
Glucose	0.65±0.03	20.2±0.7	3.11
Sucrose	0.70±0.05	28.5±0.6	4.07
D-mannitol	0.64±0.03	52.4±0.6	8.19
D-fructose	0.66±0.01	22.5±0.4	3.41
Maltose	0.65±0.008	35.5±0.5	5.46
L-arabinose	0.45±0.009	23.5±0.4	5.22
N sources			
Proteose peptone	0.65±0.01	48.8±1.5	7.50
Tryptone	0.63±0.02	49.4±0.7	7.84
Yeast extract	0.65±0.01	50.4±1.1	7.75
L-glycine	0.48±0.01	53.4±1.5	11.13
L-cysteine	0.15±0.02	0	0
$(NH_4)_2SO_4$	0.35±0.01	0.5±1.7	0.14
KNO_3	*0.55±0.01*	*52.7±0.4*	9.58

Similar results about the effect of incubation time, temperature and pH on PHB production by strain ATCC6633 were reported earlier [15]. The results of the effect of C sources on PHB accumulation by strain ATCC6633 showed that D-mannitol was most effective as C source which showed 8.2% accumulation of PHB followed by maltose with 5.5% accumulation of PHB (Table 3). However, mannitol is not recommended for industrial use because of its high cost. Therefore, PHB synthesis was often assessed in a cheaper and easily found carbon source that contained glucose culture medium instead of mannitol as a carbon source. The glucose was found optimum for PHB accumulation is some other *Bacillus* strains like Yüksekdag et al. reported that the highest PHB synthesis was found in *B. subtilis* strain 25 and *B. megaterium* strain 12 with glucose as the carbon source [10]. The production of PHB in *B. megaterium* was studied by Hori et al. and found the highest value of PHB contents when glucose was used [11]. The results of the effect of N sources on PHB accumulation showed that the maximum PHB accumulation was found in the presence of glycine as N source (Table 3). Mercan et al. also reported that PHB accumulation was high in two strains of *Rhizobium* sp. when L- cysteine and glycine were used as the nitrogen source [12]. Similar to our results were reported by Tamdogan et al [8]. The results of the three metal ions effect on PHB accumulation by strain ATCC6633 revealed that the metal ion Ni^{2+} was most effective in increasing PHB accumulation followed by Zn^{2+} and then Mn^{2+}. Ni^{2+} at 100 µM concentration level increased the PHB accumulation by 55% over control treatment where metal ions amendments were not used. Zn^{2+} at 300 µM concentration increased the PHB accumulation by 44% while this increase in case of Mn^{2+} was 30% at 150 µM concentration level over control treatment (Table 4).

Table 4: Effect of different metal ions (Zn^{2+}, Mn^{2+} and Ni^{2+}) concentrations on the accumulation of PHB by *Bacillus subtilis* ATCC6633.

Treatments	Cell dry weight (g/L)	PHB (mg/L)	PHB (%)
Control	0.65±0.02	53±0.8	8.15
Zn^{2+}			
100	1.25±0.05	58±0.5	4.64
150	0.70±0.02	62±0.6	8.86
200	0.50±0.01	66.5±0.4	13.30
250	0.45±0.008	71.5±0.8	15.89
300	0.45±0.007	76.5±0.4	16.89
Mn^{2+}			
100	0.65±0.01	66.5±0.5	10.20
150	0.80±0.02	69±0.8	8.63
200	0.25±0.01	62±1.1	13.78
Ni^{2+}			
50	0.45±0.02	68.5±1.2	15.22
75	0.55±0.01	74.5±1.7	13.55
100	0.75±0.01	82±1.3	10.93
150	0.60±0.008	66±1.1	11.00
200	0.60±0.01	64.5±1.5	10.75

The presence of metal ions has been reported to affect metabolic activities and production of different metabolites and proteins [7, 13]. Lo et al [14]. reported that Mn^{2+} enhanced the PHB accumulation in *Sphaerotilus natans* while Ca^{2+} and Mg^{2+}, reduced

the PHB accumulation in *Sphaerotilus natans*, *Pseudomonas sp. 0B17* and recombinant *Escherichia Coli.* The Cr^{3+} enhances the PHB accumulation for recombinant *E. coli.* In another report interesting results were reported that K limitation enhanced the accumulation of PHB in *Mrthylobtrcteriunl organophilum* [15]. The mechanisms of how metal ions increase or decrease the PHB accumulation are complicated and have not yet been elucidated. Many bacteria are known to synthesize a homopolyester of 3-hydroxyhutyrate, PHB, as a carbon and energy reserve material under certain environmental conditions such as the limitation of nitrogen, oxygen, or phosphate [16]. PHB has received attention as a candidate for a novel biodegradable plastic material, since it has similar physico-chemical properties to polypropylene and polyethylene. This research will serve to enhance the understanding of the effect of optimum growth conditions and metal ions on PHB accumulation by strain ATCC6633. However, more research is needed to find out efficient strains for PHB accumulation that utilize minimal resources.

References

[1] Steinbüchel A and Doi Y (2002) Biopolymers, Polyesters III, Applications and commercial product, Wiley-VCH. Weinheim

[2] Anderson AJ and Dawes EA (1990) Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev, 54 (4): 450-472

[3] Dawes EA and Senior PJ (1973) The role and regulation of energy reserve polymers in micro-organisms. Adv Microbial Physiol, 10: 135-266

[4] Jacquel N, Lo CW, Wei YH, Wu HS and Wang SS (2008) Isolation and purification of bacterial poly(3-hydroxyalkanoates). Biochem Eng J, 39(1): 15–27

[5] Braunegg G, Lefebvre G and Genser KL (1998) Polyhydroxyalkanoates, biopolyesters from renewable resources: Physiological and engineering aspects. J Biotech, 65: 127-161

[6] Poirier Y, Somerville C, Schechtman LA, Satkowski MM and Noda I (1995) Synthesis of high-molecular-weight poly([R]-(-)-3-hydroxybutyrate) in transgenic *Arabidopsis thaliana* plant cells. Int J Biol Macromol, 17(1): 7–12

[7] Raza W, Makeen K, Wang Y, Xu YC and Shen QR (2011) Optimization, purification, characterization and antioxidant activityof an extracellular polysaccharide produced by *Paenibacillus polymyxa* SQR-21. Biores Technol, 102: 6095–6103

[8] Tamdogan N and Sidal U (2011) Investigation of poly-ß-Hydroxybutyrate (PHB) production by *Bacillus subtilis* ATCC 6633 under different conditions. Kafkas Univ Vet Fak Derg, 17: 173-176

[9] Bowker RR (1981) Manual of methods for general bacteriology, American society for Microbiology. Washington DC

[10] Yüksekdag ZN, Aslim B, Beyatli Y and Mercan N (2004) Effect of carbon and nitrogen sources and incubation times on poly-beta-hydroxybutyrate (PHB) synthesis by *Bacillus subtilis* 25 and *Bacillus megaterium* 12. Afr J Biotechnol, 3 (1): 63-66

[11] Hori K, Kaneko M, Tanji Y, Xing X and Unnu H (2002) Construction of self-disruptive *Bacillus megaterium* in response to substrate exhaustion for polyhydroxybutyrate production. Appl Microbiol Biotechnol, 59(2-3): 211-216

[12] Mercan N, Aslim B, Yüksekdag ZN and Beyatli Y (2002) Production of poly-ßhydroxybutyrate (PHB) by some *Rhizobium* bacteria. Turk J Biol, 26: 215-219

[13] Raza W, Yang XM, Wu HS, Wang Y, Xu YC and Shen QR (2009) Isolation and characterisation of fusaricidin-typecompound-producing strain of *Paenibacillus polymyxa* SQR-21 active against *Fusarium oxysporum* f. sp *nevium*. Eur J Plant Pathol, 125: 471–483

[14] Lo KW, Chua H, Lo WH and Yu PH (2003) Effect of Ca^{2+}, Mg^{2+} and trace heavy metal ions on PHB-producing bacteria. Poster Presentation 6-27. www1.eere.energy.gov/…otech_symposium/docs/6-27.doc

[15] Kim SW, Kim P, Lee HS and Kim JH (1996) High production of poly-P-hydroxybutyrate (PHB) from *Methylobacterium organophilum* under potassium limitation. Biotechnol Lett, 18 (12): 2530

[16] Braunegg G, Lefebvre G and Genser KL (1998) Polyhydroxyalkanoates, biopolyesters from renewable resources: Physiological and engineering aspects. J Biotech, 65: 127-161