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Lab-Scale Optimization of Polyhydroxyalkanoate Production by Bacterial Strain cmg1415 on Local Cheap Substrates Using One Variable at a Time Approach

Muhammadi*, Shabina Shafiq

Centre for Bioresource Research, Islamabad, Pakistan.

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ABSTRACT

Production of polyhydroxyalkanoate (PHA) under optimum culture conditions using local cheap feedstocks is indispensable to overcome the current cost of PHA-based plastics. For this purpose, optimum culture conditions and cheap feedstocks were investigated to produce maximum yield of PHA in CMG1415. Maximum yield was obtained with sucrose or sugar beet as sole source of precursors for PHA in 8 days of incubation at 35 °C in a minimal medium adjusted at pH 7. Further, for maximum yield no mechanical shaking was needed. Local cheap feedstock such as sugar beet and molasses were found to play as significant carbon and nitrogen sources for maximum PHA yield. Bacterial plastic produced under these low-labor-cost culture conditions may to reduce the present cost of degradable bioplastic and be much effective alternate of nondegradable varieties of synthetic plastic.

Corresponding Author: Muhammadi
Email: muhammadi12@yahoo.com

INTRODUCTION

The Polyhydroxyalkanoates (PHAs) are biodegradable polyesters of hydroxyalkanoates (HAs) synthesized by numerous bacteria as intracellular energy reserve compounds when bacteria must survive under unfavorable conditions (Burdon, 1946; Kunioka *et al.,* 1989; Anderson *and* Dawes, 1990; Pfeffer 1992; Sang, 1996). They act as an energy storage facility and are developed when the bacteria's surroundings include excess carbon, and a deficiency of another nutrient e.g., oxygen or nitrogen source limitations (Poirier *et al.,* 1995; Steinbuchel, 1991; Lee and Chang, 1995; Tina *et al.,* 2001; Salehizadeh and Loosdrecht, 2004). After complete utilization of the nitrogen in the nutrient broth, the bacteria can no longer grow, and energy derived from the sugar in the medium is used for the production of the reserve material. Bacterial PHAs have physical properties similar to those found in traditionally used non-biodegradable, petrochemical-derived thermoplastic polyethylene and polypropylene. Unlike commonly used synthetic plastic, bacterial PHAs are biodegradable and biocompatible. Hence, PHAs is widely regarded as a potential replacement of certain traditional thermoplastics that constitute a persistent post-consumer waste (Tamer *et al.,* 1998). Approximately 80 years ago, the first PHA polymer PHB was isolated from a *Bacillus magaterium* bacteria cell. Since that time, biopolymer scientists have been attempting to find ways to expand and commercialize bacterial production of biopolymer materials (Wong *et al.,* 2000). Microbial production of all PHAs is expensive, thus those polymers are used at present only as specialty plastics. Significant contributors to cost of production are the fermentation processes using various materials as feedstock materials (Poirier *et al.,* 1995; Choi and Lee, 1997) and downstream processing (Berger *et al.,* 1989; Lee and Chang, 1995). In a study by Wong *et al.,* (2000), pure fructose, barley malt, and even sesame oil was found to be suiTable fermentation feedstocks for PHB produced by a bacterium (*Staphylococcus epidermis*) isolated from sesame oil

processing waste. Throughout the 1990's, researchers at McGill University worked on developing a process by which the waste from potato chip processing could be used to microbially produce PHB (Rusendi and Sheppard 1995). Bacterial PHAs production also depends on growth conditions that can be optimized to achieve a reliable yield. (Kunioka, 1989; Lee and Chang, 1995; Omar *et al.,* 2001). Manchak *et al* (1995) and Silva *et al.,* (2004) scaled up the PHA production using cheap carbon and nitrogen sources. Inexpensive and scaleable recovery schemes need to be devised to achieve low-cost production that is competitive with traditional thermoplastics (Tamer *et al.,* 1998). The aim of present study is to optimize the PHA yield at lab-scale using local cheap carbon and nitrogen sources at optimum growth condition.

METHODS AND MATERIAL

Bacterial Strains and Media

A minimal medium, used for bacterial PHA production contains following constituents in 100ml distilled water: 2 g carbon source (Fructose, Glucose, Sucrose, Na-gluconate) (separately autoclaved at 110 ^{o}C for 15 minutes), 0.68 g KH_2PO_4, 0.88 K_2HPO_4, 0.02 g $MgSO_4.7H_2O$, 0.01 g NaCl, 0.05 g Yeast Extract, 0.05 Urea (filtered by millipore filter, 0.22 µm). Urea was used as an inorganic nitrogen source, and yeast extract was used as an organic nitrogen source. PHA producing bacterial strain CMG1415 an unidentified soil gram positive bacillus was selected from CMG culture stock and grown on agar plates of Brain Heart Infusion (BHI) at 37 °C overnight. Single isolated bacterial colony was in above minimal medium adjusted at pH 7 and then incubated at 30 ˚C for 24hrs as seed culture for downstream optimizing experiments.

Optimization of Culture Conditions

Culture conditions (pH of medium, incubation period and temperature) and carbon sources were optimized using a "one-variable -at-a-time" (OVAT) approach (Van den Berg *et al.,* 1995). 1 ml of 24 hrs old seed culture was inoculated into 100 ml of above mentioned medium adjusted at pH (4, 5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 and 10) and incubated at different temperatures (10, 15, 20, 50 ˚C) for different stipulated incubation periods (1-10 days). In one set of experiment bacterial strain was grown statically while in same set of another experiment was subject to shaking (40, 60, 80 and 100 rpm) throughout our experiments. For each set of experiment, medium was supplemented with 2 % of either one of carbon source (Fructose, Glucose, Sucrose, Na-gluconate, 5 g sugar beet, 4ml molasses and 25 g sugar can bagasse) to be optimize. Sugar beet (40-45 % sucrose), molasses (45-50 % sucrose) and sugar cane bagasse (8-10 % sucrose) were used as local cheap carbon and nitrogen sources. After each stipulated incubation period the growth of culture was terminated and intracellular PHA was extracted from cells. Effect and role of nutrients (carbon sources, urea, and yeast extract) in PHA production were investigated with supplementation and absence.

Extraction of PHA

Bacterial cells were collected by centrifugation at 10,000 rpm for 20 minutes in refrigerating centrifuge (4 °C) and washed with 0.89 % NaCl solution. Intracellular PHAs were extracted from cells with excess of chloroform (15 ml/g biomass, 25 °C, 48 hours) (Kim *et al*., 1991; Kunioka *et al.,* 1989). Residual cell material was removed by filtration (0.4 µm), the polymer formed was precipitated by addition of chloroform solution into cold methanol (1:10 v/v) (Kim *et al.,* 1991; Page *et al.,* 1993; Mun *et al.,* 1995) and precipitated polymers were washed with methanol and dried in Wheaton dry seal desiccator over $CaCl_2$ to a constant weight. Quantification of PHA produced was made according to spectrophotometric method of Law and Slepecky (1961) in triplicate and average amount PHA was calculated.

Statistical analysis

The results were analyzed statistically using the Statistix 8.1 software. The means of four repeated experiments were compared using one way ANOVA and result was considered significant followed by Tukey HSD test at $P<0.05$ significance level.

RESULTS

Role of Nutrients on PHA Production

It was shown in Table 1 that a considerable yield of PHA was obtained only in the presence of carbon source. In case of supplementation of only yeast extract a trace amount of PHA was obtained. Without combination of carbon and yeast extract, urea itself could not give PHA. Combination of three sole nutrients was found to give a significant yield among all combinations as indicated by asterisk in Table 1. Substitution of urea with ammonium chloride was found to give a comparatively reduced amount of PHA (Table 1).

Table 1. Role of some nutrients on production of PHA in CMG1415.

Description	PHA mg/100ml of culture
C. S^+/ U^-/ Y. E^-	35.634
C. S^-/ U^+/ Y. E^+	0.751
C. S^-/ U^+/ Y. E^-	0
C. S^-/ U^-/ Y. E^+	0.553
C. S^+/ U^+/ Y.E^-	40.325
C. S^+/ U^- / Y.E^+	39.356
C. S^+/ U^+/ Y. E^+	48.252*
C. S^-/ A^+/ Y. E^+	0.675
C. S^-/ A^+/ Y. E^-	0
C. S^+/ A^+/ Y.E^-	37.453
C. S^+/ A^+/ Y. E^+	44.345

C.S: Carbon Source, U: Urea, Y.E: Yeast Extract, A: Ammonium chloride, $^-$: Absent, $^+$: Present, *: maximum yield at certain condition.

Optimum Carbon Source for maximum PHA Production

Figure 1 showed that in combination with urea all carbon sources were found to give better yield as compared to that without urea. PHA yield obtained from local cheap feed stocks in case of without urea was found to be comparatively equal to that from with urea. Among carbon sources, maximum yield was obtained with sucrose-urea combination while that was obtained from sugar beet without urea. Na-gluconate was found to poor carbon source (Figure 1).

Optimum Incubation Period for the PHA Production

Biosynthesis of PHA was detected even in 24hr of incubation (Figure2). An increasing pattern of PHA yield was obtained up to 8^{th} of incubation while after 8^{th} day, a slow rate of reduction was observed. The maximum yield was obtained in 8 days of incubation as shown in Figure2.

Optimum pH for maximum PHA Production

The maximum yield of PHA was extracted from cells grown in medium adjusted at pH 7 as signified by asterisk in Table 2. At below and above the neutral pH a reducing pattern of yield was extracted. Bacterial strain could not show any observable growth at pH 4 and10.

Optimum Temperature for the PHA Production

Results in Figure3 showed that PHA was obtained even at 10 °C of incubation temperature. As temperature rose, proportionally increasing PHA yield was extracted up to 35 °C but after that an observable reduced quantity of PHA was found to obtain. The maximum quantity was obtained at 35 °C as shown in Figure3.

Table 2. Optimum pH of medium for production of PHA in CMG1415.

pH of medium	PHA yield produced (mg/100ml)
4	-
5	12.552
6	23.57
6.5	29.878
7	48.553*
7.5	41.675
8	27.44
8.5	21
9	12.24
9.5	5.544
10	-

- : No growth, *: maximum yield at certain condition.

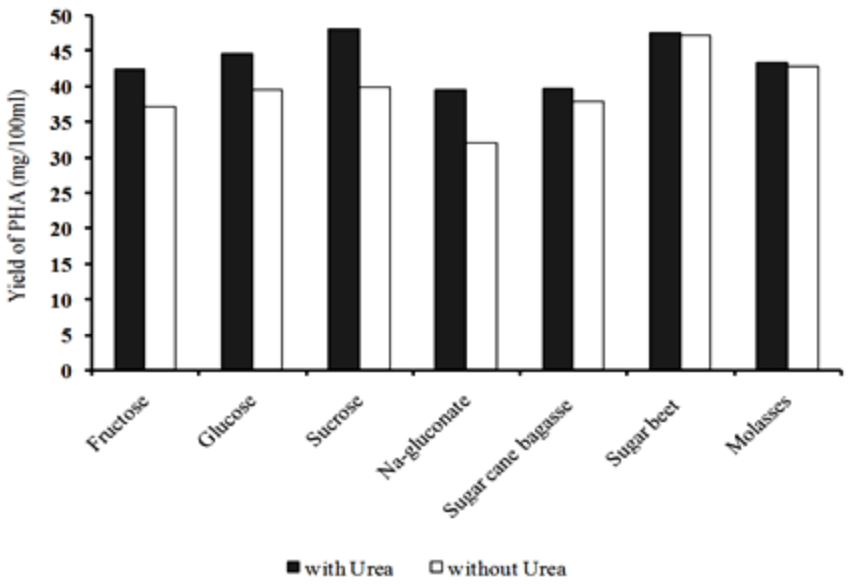

Figure 1. Optimization of carbon source for the maximum production of PHA by CMG1415 under optimum culture conditions.

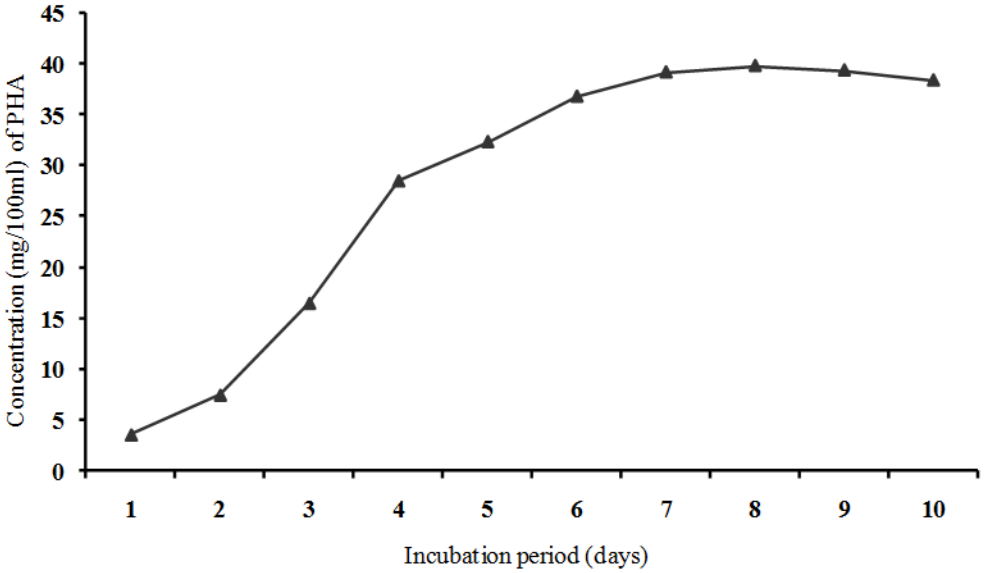

Figure 2. Optimization of incubation period for the maximum production of PHA by CMG1415.

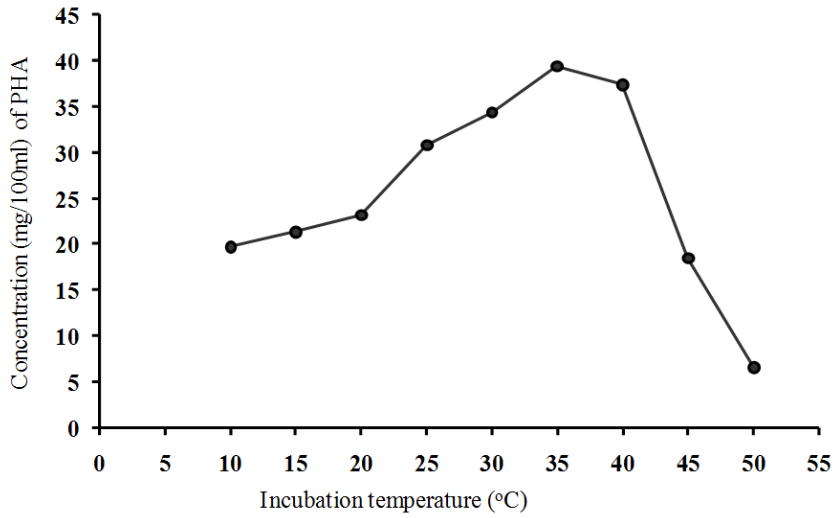

Figure 3. Optimization of incubation temperature for the maximum production of PHA by CMG1415.

DISCUSSION

In the broad view of its wide rang industrial applications PHA production in CMG 1415 was when quantified under certain culture conditions it was found that it is culture conditions dependent. In the result of shaking culture conditions, a negligible yield of PHA was obtained and satisfactory results were observed only in static condition. Therefore, it is suggested that for the production of PHA in CMG1415 any expensive and especial shaking instrument or mechanical method is not required, so it is simple. CMG1415 could not produce PHA with inorganic nitrogen source only and synthesis was observed in the presence of carbon source suggesting that for PHA production precursors are provided solely from carbon source. Nitrogen sources were found to enhance the production in the presence of carbon source (Table 1). Although among carbon sources maximum yield was obtained from combination sucrose with nitrogen source as spectrophotometrically estimated. But among tested carbon sources, from sugar beet and molasses without addition of nitrogen source a significant yield was obtained. This suggested that sugar beet and sugar cane bagasse contain usable nitrogen source as they were extracted from living tissues (Poirier *et al.,* 1995; Sang, 1996; Silva *et al.,* 2004). Bacterial strain CMG1415 required 8 days of incubation period for maximum yield of PHA synthesis and accumulation (Figure 2). After 8th day, an observable reduced amount was obtained due to for prolong incubation (9 and 10 day) synthesized amount was biologically depolymerized into simple carbon source for bacterial use which is a common phenomenon among bacterial flora (Kunioka *et al.,* 1989; Jendrossek *et al.,* 1996; Saruul *et al.,* 2002). From Figure 3 it was suggested that at both low (<25^{o}C) and high (>40^{o}C) temperatures, the rate of PHA biosynthesis became slow down and the optimum temperature for maximum yield lie between the ranges of 30-37 ^{o}C. However, tested bacterial strain produced maximum yield at 35 ^{o}C which may be the selective temperature suitable for activation of monomers into precursors, polymerization of precursors into PHA, and its accumulation in cell. A number of researchers have reported that optimum incubation temperature for the production of biodegradable plastic (PHA) lied between the range of 28-37 ^{o}C among PHA producing bacterial species (William *et al.,* 1989; Rhee *et al.,* 1993; Gerrit *et al.,* 1995; Seon, 1996). CMG1415 could not grow in high acidic and alkaline media therefore, produced a gradually reduced PHA yield could be extracted from cells grown in acidic and basic media due to poor growth (Table 2). Satisfactory yield was obtained only from cells grown in media adjusted at pH 7 which is same to the previous studies of many research groups (Gerrit *et al.,* 1995; Manchak *et al.,* 1995; Mun *et al.,* 1995). These results have shown that PHA production is influenced by culture condition like incubation period, temperature, carbon, and nitrogen sources. Therefore, it is concluded that for maximum PHA yield from CMG1415, the culture system

should obey neutral medium containing sucrose carbon source or sugar beet or molasses as local cheap feedstock, 35 ^{o}C incubation temperature and 8 days incubation. Therefore, it is concluded that under these cheap and optimum conditions the yield of bacterial plastic can be considerably enhanced to develop and promote the plastic industry. Thus, can be replaced the nondegradable currently available commercial plastic with biodegradable bacterial plastic.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS CONTRIBUTIONS

All the authors contributed equally to this work.