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One-Factor-at-a-Time Optimization of Polyhydroxybutyrate Production and Growth of Alcaligenes eutrophus by Altering Culture Parameters and Incubation Time

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Abstract

Polyhydroxyalkanoates (PHAs) are bioplastics derived from renewable resources such as vegetable oils, corn starch, or microbes. The polyhydroxybutyrate (PHB) is a short-chain-length PHA, and the most important bioplastic produced by certain microorganisms in the presence of excess carbon sources. In this study batch cultivation of Alcaligenes eutrophus with the aim of increasing PHB production using different carbon and nitrogen sources was performed. The accumulation of PHB granules in this strain was significantly dependent on the type of carbon and nitrogen sources in the culture medium. The bacteria were cultivated on various carbon sources including glucose, fructose, lactose, lactic acid, arabinose and sucrose and nitrogen sources including ammonium chloride, ammonium sulfate, peptone, urea and tryptone at constant concentrations, temperature and pH. Cell growth and PHB production were quantified by measuring absorbance at 600 nm and 235 nm (absorbance of crotonic acid), respectively. The best results were obtained when using fructose and ammonium chloride as carbon and nitrogen sources, with a carbon/nitrogen ratio of 10. The production of PHB was growth associated as indicated by the growth and PHB production kinetics. Atomic force microscopy analysis of PHB film also showed high porosity of PHB recovered by chloroform.

Keywords: Alcaligenes eutrophus; Carbon source; Incubation time; Polyhydroxybutyrate; Nitrogen source.

Introduction

Plastics are one of the most widely used materials in the world, and have enormous applications in agriculture, medicine and food industry. These materials are not biodegradable and because of their resistance to microbial degradation, they get accumulated in the environment and their disposal poses serious threat to the environment. These facts have stimulated interests in biodegradable polymers such as PHAs as alternatives

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to conventional plastics [1,2].

PHB is a short-chain-length PHA which can accumulate up to 90% of dry cell weight in some gram-negative bacteria in the form of intracellular granules [3]. *Alcaligenes eutrophus* is the most important bacteria for the production of PHB due to high efficiency and high PHB production rate using simple carbon sources. These granules act as energy reserve materials in limiting concentrations of nutrients such as nitrogen and phosphorous sources and excess amounts of carbon sources [4, 5]. Until the 1980s, this compound was not used as substitute for petroleum-based plastics. In the 1990s, the interest in biomedical industry became popular and research in biodegradable plastics has become steady and profitable. So far, researchers have invented several different types of bioplastics and a variety of manufacturing methods [6].

Many studies have shown that the yield of PHB production, depend on different culture conditions and the type of nutrients, particularly carbon and nitrogen sources [7, 8]. So far different experiments have been conducted and the effect of various parameters such as temperature, pH, agitation speed, carbon, nitrogen, phosphorus, and mineral elements on accumulation of PHB have been studied in different bacteria [7-14]. For example, Khanna and et al. [7] determined that fructose, lactic acid, sucrose and glucose are the best carbon sources for *A. eutrophus* growth and PHB production in the mineral salt medium. They suggested that using fructose and ammonium sulfate as carbon and nitrogen sources could increase PHB concentration up to 1.4 g/L in the 48 h batch culture. They also showed that the highest amount of PHB production can obtain by using urea as the nitrogen source.

It is obvious that the first step in the production of each metabolite is optimizing different parameters affecting culture production in batch culture, before the fermentation processes. In the present study, efforts have been made to determine the best carbon and nitrogen sources and incubation time for optimized production of PHB in *A. eutrophus* using a one-factor-at-a-time methodology. The results can be used for the maximum production of PHB in future experiments. The pH changes of media during cultivation time were also investigated. Finally, characterization of PHB films was done by AFM in order to acquire knowledge about its structure for further application.

Materials and Methods

Microorganism

Alcaligenes eutrophus PTCC 1615 was obtained from the Persian Type Culture Collection for PHB production. The PHB producing capability of the organism was confirmed by Sudan Black staining method. The bacteria were maintained on LB agar slants at 4 ºC. For inoculums preparation, the cells were grown on a LB medium for 10 h at 30 ºC on a shaker at 200 rpm. The compositions of the LB medium were (g/L): peptone 10, yeast extract 5.0, and sodium chloride 5.0. Stock cultures were maintained at -20 ºC in 5-ml vials containing 4 ml of basal medium and 1 ml of glycerol solution until use.

Media

Because *A. eutrophus* grow under aerobic condition, in all cases, the medium size was one-fifth of the flasks volume.

The composition of the basal mineral salt medium (MSM) used in this study was as follow: $(NH_4)_2SO_4$ 2.0 g/L, KH_2PO_4 1.54 g/L, $MgSO_4.7H_2O$ 1.2 g/L, Citric acid 1.7 g/L, trace metal solution 10 mL/L [15]. The trace element stock solution composed of $ZnSO_4.7H_2O$ 2.25 mg/L, $FeSO_4.7H_2O$ 10 mg/L, $CaCl_2(2H_2O)$ 2 mg/L, $Na_2B_4O_7(7H_2O)$ 0.23 mg/l, $(NH_4)_6Mo_7O_{24}$ 0.1 mg/L, $CuSO_4$ $(5H_2O)$ 1 mg/L, $MnSO_4(5H_2O)$ 0.6 mg/L, and HCl 35% 10 mL/L [16]. Fructose was used as carbon source in concentration of 20 g/L for production media and inoculum development. The initial pH was adjusted to 7.0. The inoculum size for the all experiments was 10%.

Comparison of different carbon and nitrogen sources carried out using the media as described above. However, for comparison, different carbon (20 g/L) and nitrogen (2 g/L) sources were taken.

Growth and cell dry weight determination

Cell growth was monitored over time by measurements of optical density at 600 nm (OD_{600}) using jenway 6310 (Bibby Scientific Limited, Staffordshire, UK).

Cell dry weight determination was done by weighing the cell dry mass obtained as follows. 10 ml culture samples were centrifuged at 10000 rpm for 15 min at 4 ºC. The pellet was resuspended in distilled water (10 ml) and centrifuged again for washing. The washed cells were dried at 90 ºC for 24 h in a hot air oven. The drying was repeated until constant weight was obtained [17].

Detection of PHB

To analyze the amount of PHB, 10 mL of 98% sulfuric acid was added to bacterial pellet at 90ºC for 1 h; PHB crystals in cytoplasm of bacteria were converted into crotonic acid. The absorbance of the solution was measured at 235 nm in a UV spectrophotometer. The

amounts of PHB per gram (dry weight) of bacterial cells were determined using a standard curve.

Effect of different carbon and nitrogen sources on growth and PHB production

The experiments were performed by one-factor-at-a-time methodology (OFAT). OFAT is a method of designing experiments involving the testing of factors, one at a time instead of all simultaneously. These experiments were first carried out in MSM medium containing different carbon sources (20 g/L) (*i.e.* fructose, glucose, lactic acid, arabinose, sucrose, and lactose) or at second-stage MSM containing fructose (20 g/L) and various nitrogen sources (2 g/L) (*i.e.* ammonium chloride, ammonium sulfate, peptone, tryptone, yeast extract and urea). The flasks were incubated at 30 ºC and 200 rpm. Inoculum was prepared as described above.

PHB recovery for film preparation by using hypochlorite and chloroform

One gram of cell powder was treated with dispersions of 50 ml chloroform and 50 ml hypochlorite solution 10%.

After treatments at 37 ºC for 1 hour, the dispersion was centrifuged. Three separate phases were obtained. The upper phase was hypochlorite solution, the middle phase contained some undisrupted cells among others, and the bottom phase was chloroform containing PHB. Hypochlorite phase was removed first, and then the chloroform phase was obtained by filtration. After the filtration, PHB was recovered by non-solvent precipitation and filtration.

Atomic Force Microscopy of PHB Films

Microphotographs of the surface of PHB films were obtained be means of atomic force microscopy (AFM). The AFM imaging was performed with Solver PRO-M (Zelenograd, Russia). For AFM imaging a piece of the PHB film (~2.2 mm^2) was fixed on a sample holder by a double-sided adhesive tape. Silicon cantilevers NSG11 (NT-MDT, Russia) with typical spring constant of 5.1 N/m were used. The images were recorded in semi-contact mode, scanning frequency of 1–3 Hz, scanning areas from 3.3 to 20.20 μm^2, topography and phase signals were captured during each scan. Image processing was carried out using DME-SPM software.

Results and Discussion

The rate of cell growth and PHB production at the basic level

At initial experiments, fructose was used as carbon source in concentration of 20 g/L. The cell growth and production of PHB were described as a function of the cell dry weight (biomass), OD_{600}, pH, and the PHB at the basic level (Fig. 1). From cell dry weight (biomass) and OD_{600} results, it can be found that the bacterial growth increase continuously and reach to the maximum amount of 1.375 g/L after 72 h. The results of PHB analysis showed that the maximum content of PHB (0.015 g/L/h) and the efficiency of 73%, is obtained after 48 h. After this time a slightly decrease in PHB amount can be observed. This experiment showed

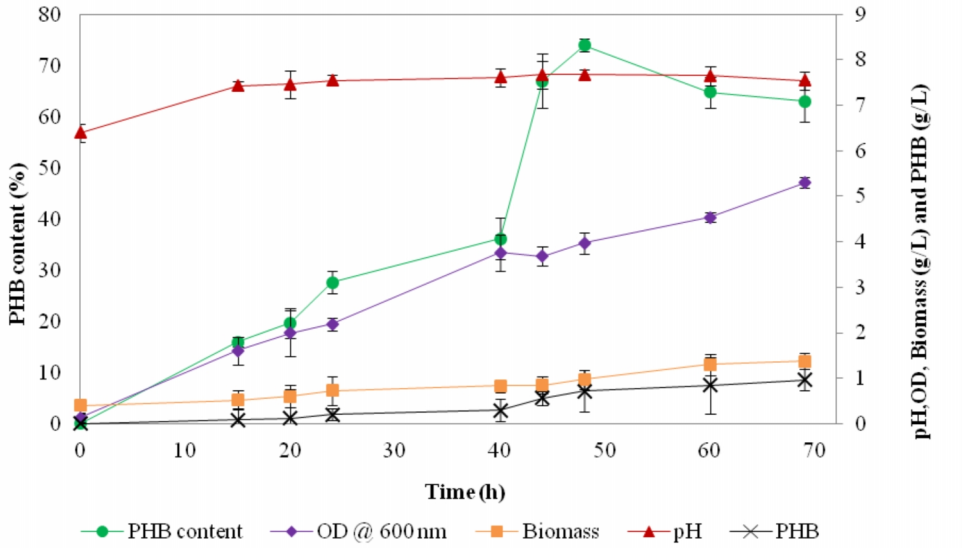

Figure 1. pH, Biomass, PHB and absorbance during 72 h culture of *Alcaligenes eutrophus*.

that 48 h is sufficient for harvesting cells, because the maximum PHB yield is during this time of cultivation.

These results are in accordance with Khanna and et al. studies in batch mode using *A. eutrophus*. They reached to the maximum PHB production and biomass of 0.031 g/L and 2.332 g/L, respectively after 48 h using glucose as a carbon source (40 g/L), and 1.4 g/L and 3.25 g/L using fructose as carbon source (40 g/L). This reduction in PHB production after 48 h may be due to lack of micronutrients as well as increase in metabolites that might have negative effect on the PHB production [18]. Studies conducted by Bonartseva et al. [19] are also in consistence with these results wherein the maximal PHB accumulation was observed at 48 h. After 48 h, unfavorable conditions of the medium caused a decrease in PHB yield. This might be because of increase in medium viscosity as a result of exopolysaccharide production and limitation of oxygen transfer [20]. Although dry cell weight increased after 48th h, the decrease of PHB might indicate that the bacteria used PHB as a source of carbon and nitrogen, causing an unsuitable condition due to inadequate nitrogen and carbon sources in the medium.

Aslim et al. [22] observed that the PHB production in *Rhizobium* sp. strain was maximum at pH 7.0. Grothe et al. [23] also reported that pH value ranging from 6.0–7.5 is optimum for PHB production. Therefore in this study initial pH was adjusted at 7. From Figure 1 it can be seen that pH values didn't change significantly during 72 h culture and the medium had relatively strong buffering capacity. Khanna et al. experiments showed high decrease in pH from 7 to 5.26 after 48 h [7]. In their experiments, decreasing pH led to the adverse conditions and thus reduces growth and nutrient intake. This difference may be due to lower level of carbon source used (20 g/L) in this study compared to previous experiments, because several studies show that the consumption of sugar caused by various bacteria produce compounds, which leads to a decrease in medium pH [21].

Effect of different carbon sources on growth and PHB production

The cell dry weight, pH values, OD_{600}, and PHB production of the *A. eutrophus* were determined using various carbon sources including glucose, fructose, arabinose, lactose, lactic acid and sucrose that coverage the constituents of different cost-effective waste materials. Finding appropriate carbon source for *A. eutrophus*; give us insight in to using which cost-effective carbon sources (*i.e.* palm pulp, date extract, molasses, and whey) is suitable at industrial scale. Study and design of experiments was according to one-factor-at-a-time methodology using Design-Expert 7.0.0 (Stat-Ease, Inc., Minneapolis, MN, USA) software. Each experiment was repeated two times, resulting in 12 trials for this step. Experiments were performed in 250 ml flasks containing 50 ml of MSM, the inoculation size was 10%, and the temperature and agitation speed were set to 30 ºC and 200 rpm, respectively. Samples (1.0 ml) of culture medium were withdrawn every 2 h and the amount of pH, biomass and PHB were measured.

All the commercial carbon sources supported good growth of the bacterium as well as the production of PHB. *A. eutrophus* had the highest biomass yield of 6.43 g/ L of culture in glucose, followed by 6.08 g/L in fructose, 5.88 g/L in lactose, 5.32 g/L in lactic acid, and 3.55 g/L in sucrose while arabinose had the least biomass yield of 2.91 g/L (Fig. 2). The results show that in all experiments, the biomass production rate has increased until about 48 h but from 24-48 h this increase markedly slow down probably due to unfavorable

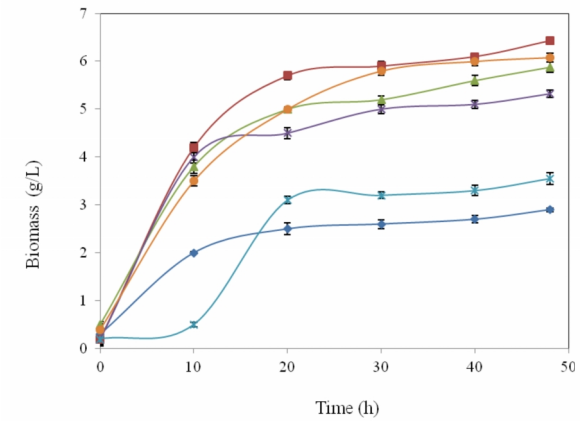

Figure 2. Effect of different carbon sources on the biomass of *A. eutrophus* in Arabinose (♦), Fructose (●), sucrose (*), lactic acid (×), lactose (▲), Glucose (■).

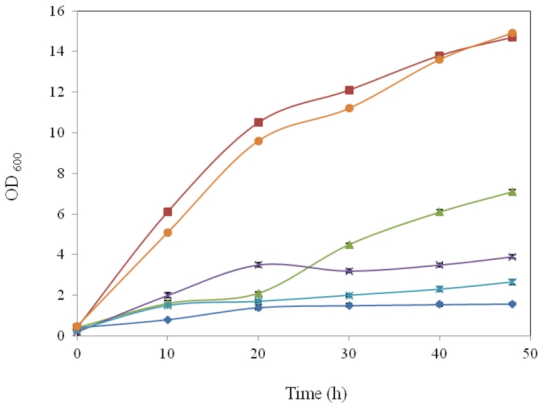

Figure 3. Absorbance changes for different carbon sources during 48 h. Arabinose (♦), Fructose (●), sucrose (*), lactic acid (×), lactose (▲), Glucose (■).

growing conditions. We can obtain better biomass yields in the presence of 6 and 12-carbon sugars (*i.e.* lactose, fructose, glucose and lactic acid), compared to the 5-carbon sugars. Probably because this strain is six-carbon sugars utilizing bacteria [25].

Analysis of OD_{600} (i.e. the optical density and cell growth) is shown in Figure 5. Fructose containing medium had OD_{600} of 14.98 after 48 h following glucose (14.73), lactose (7.1), lactic acid (3.9), sucrose 2.65, and arabinose 1.58. The results show that using glucose and fructose as carbon sources yield higher turbidity representative of good growth and PHB production.

The pH values of the culture medium ranged from 7 to 8.2 ± 0.33 for fructose, 7 to 8.2 ± 0.07 for lactic acid, 7 to 8.1 ± 0.06 for glucose, 7 to 7.5 ± 0.2 for arabinose, 7 to 7.93 ± 0.02 for sucrose and 7 to 8.28 ± 0.04 for lactose (Fig. 4). As seen the changes in pH values was from 7 to 8.5 for all carbon sources except arabinose. In general we can say that pH did not change significantly during cultivation time.

The highest PHB production of *A. eutrophus* recorded within 48 hours from fructose medium with production of 2.08 g/L, glucose (1.32 g/L), lactic aicd (0.63 g/L), lactose (0.28 g/L), and sucrose (0.25 g/L) while arabinose has a production of 0.10 g/L (Fig. 5). Therefore, the maximum PHB production and biomass has been achieved by fructose and glucose, respectively. Although the highest biomass was obtained from glucose, as compared to fructose, the PHB content was less. Because biomass production in glucose and fructose was near and PHB production in fructose was higher, fructose was selected as carbon source for further studies.

Working with different carbon sources in MSM broth, Khanna and Srivastava [7] observed higher PHB yield on fructose by *Alcaligenes eutrophus*. Propionic acid, gained the lowest yield of PHB production and biomass, probably due to the inhibitory effect of high concentrations of this acid. They reported that glucose and fructose, being monosaccharides were readily utilized by bacteria and, hence, support growth and subsequently PHB production; however, the complex molecules like lactose were not utilized for effective PHB production. In our experiments also, lactose and sucrose had the lowest rate of PHB production. This is probably due to the lack of proper enzymes in *A. eutrophus* to break down these sugars and making full use of them as carbon sources [26]. Naturally occurring strains of *A. eutrophus* has also been reported to utilize only fructose as carbon source [27]. As the complexity of the carbon source increased, PHB yield was found to decrease. Similar conclusions were made by Joshi and Jayaswal [28].

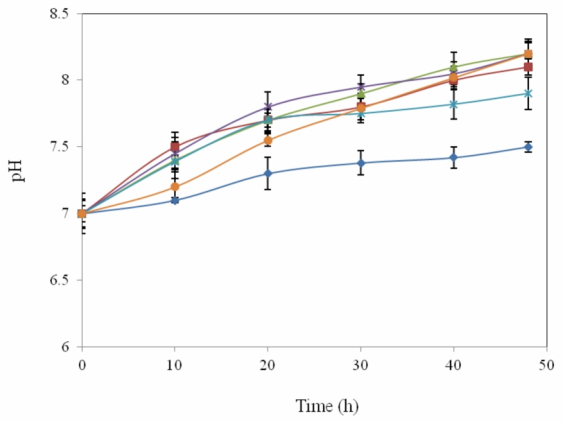

Figure 4. pH variations in the culture media during cultivation of *A. eutrophus* in Arabinose (▲), Fructose(■) , Lactose (♦) , Sucrose (●), Lactic acid (×), Glucose (-) .

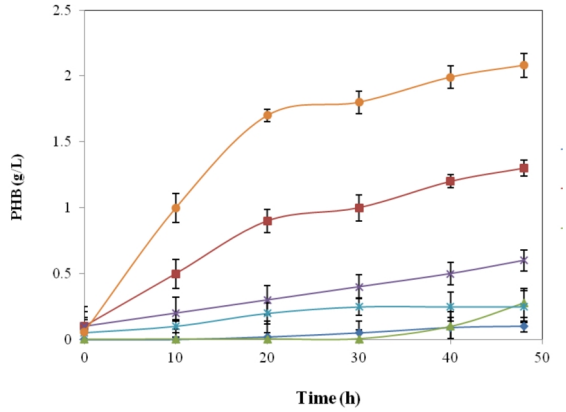

Figure 5. PHB concentration changes for different carbon sources during 48 h. Arabinose (♦), Fructose (●), sucrose (*), lactic acid (×), lactose (▲), Glucose (■).

Effect of different nitrogen sources on growth and PHB production

The effects of different nitrogen sources on the growth and PHB production are shown in Figures 6–9. Six different nitrogen sources were selected from literature at concentration of 2 g/L including: peptone, tryptone, yeast extract, urea, ammonium sulfate and ammonium chloride. In this study, PHB accumulation was induced by phosphate limitation instead of nitrogen limitation [29].

Experiments were done in 250 ml Erlenmeyer flask containing 50 ml of MSM with the same conditions as the previous experiments. Concentration of fructose was 20 g/L.

A. eutrophus grew best in media containing ammonium chloride as nitrogen source, probably due to its simple structure that can be easily used by this strain.

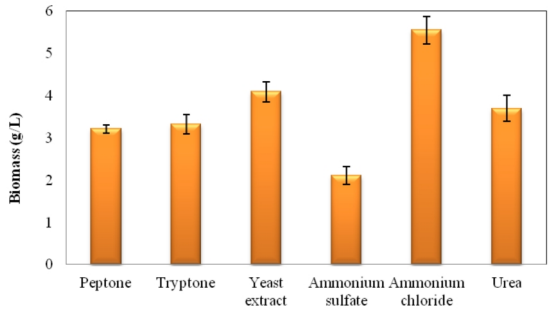

Figure 6. Cell dry weight for different nitrogen sources during 48 h.

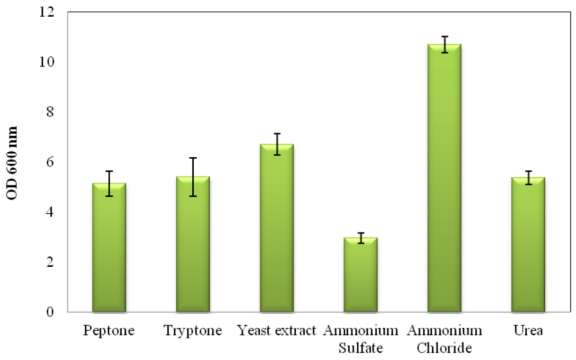

Figure 7. OD_{600} for different nitrogen sources during 48 h.

Ammonium chloride had the maximum biomass growth of 5.55 g/L, yeast extract 4.09 g/L, urea 3.45 g/L, tryptone 3.33 g/L, peptone 3.21 g/L and ammonium sulfate with a value of 2.1 g/L of the culture medium (Fig .6). The least biomass yield was observed in the presence of ammonium sulfate. This in contrast to study of Koutinas et al. that reported ammonium sulfate is the best nitrogen source for PHB production of *A. eutrophus* [30].

Analysis of OD_{600} of *A. eutrophus* in the various nitrogen substrates is shown in Fig.7. Ammonium chloride had the highest OD_{600} of 10.68 within 48 hours, yeast extract 6.7, tryptone 5.4, urea 5.38, peptone 5.14, and ammonium sulfate 2.97 (Fig. 7). The medium absorbance for the all nitrogen sources except ammonium sulfate can be said to be in a similar range.

The pH values of the culture medium within 48 h ranged from 7 to 8.32 for peptone, 7 to 8.25 for tryptone, 7.0 to 8.24 for urea, 7 to 8.1 for yeast extract, and 7 to 8.005 for ammonium sulfate, and 7 to 5.18 for ammonium chloride (Fig. 8). It can be seen that except ammonium chloride the other sources did not change pH of culture medium significantly. pH values in the presence of ammonium chloride decrease significantly,

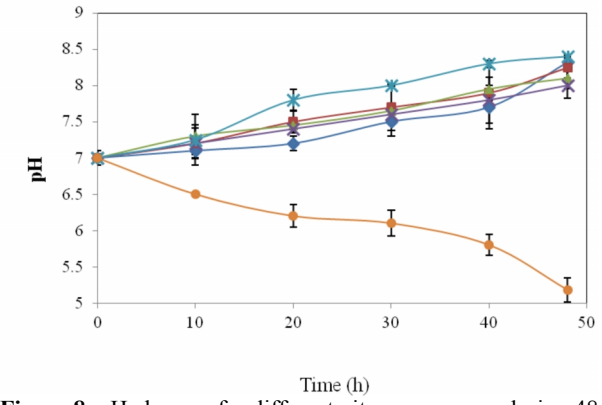

Figure 8. pH changes for different nitrogen sources during 48 h. Yeast extract (▲), Peptone (♦), Tryptone (■), Ammonium chloride (●), Urea (*), Ammonium sulfate (×) .

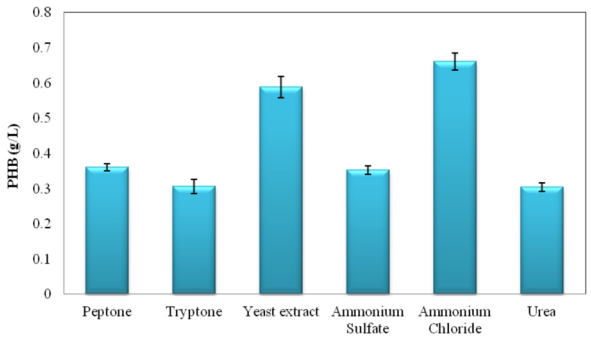

Figure 9. PHB production for different nitrogen sources during 48 h culture.

but it seems that it did not greatly affect growth or production of PHB by the *A. eutrophus*.

The highest PHB production per nitrogen sources are as follows: ammonium chloride medium 0.66 g/L, yeast extract 0.58 g/L, peptone 0.36 g/L, ammonium sulfate 0.35 g/L, and tryptone and urea 0.30 g/L, respectively, within 48 hours, (Fig. 9).

It can be concluded that in the presence of ammonium chloride and yeast extract maximum level of PHB production is obtained. The only problem in selecting ammonium chloride as nitrogen source is decreasing of pH during cultivation time. For increasing PHB production, pH should be maintained using the controller. Here, the reduction of PHB does not cause the reduction of biomass which is in contrast to results of Repaske who reported that (in the absence of pH controller) at an ammonium concentration higher than 0.5 g/l, the growth of *A. eutrophus* rapidly stopped when the pH dropped below 5.4 [31,32].

Khanna and et al. were also investigated the effect of different nitrogen sources on PHB production. They found that urea, ammonium sulfate, ammonium chloride and ammonium nitrate were the best nitrogen sources,

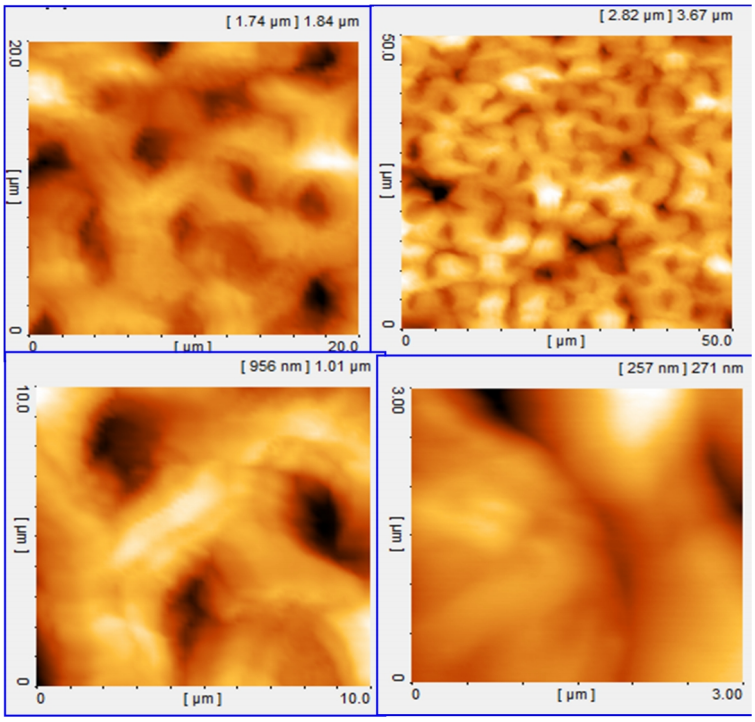

Figure 10. AFM topographic images of PHB films. Scan sizes are shown on top of each picture. The rough surface of fresh-prepared sample (exposed to air) is seen.

respectively [7]. Raje and Srivastav [33] and Mulchandani *et al.* [34] also studied the accumulation of PHB by *A. eutrophus* with different salts of ammonium. Similar to the results of the present study, they also obtained highest PHB yield in ammonium sulfate or ammonium chloride. Ammonium chloride is a simple nitrogen source and probably more readily available than the complex nitrogen sources such as yeast extract. This is in contrary to the results of Page who suggested that using complex nitrogen sources (e.g. fish peptone, protease peptone, yeast extract, phytone and tryptone) leading to increased efficiency of the PHB production in *Azotobacter vinelandii* [35].

The results of the present study are in accordance with Khanna and Srivastava [7], Belal [36], and Shaaban et al., [37] who have reported maximum PHB production at 2 % concentration of nitrogen source. It was found that Carbon/Nitrogen ratio as 10:1 resulted into maximum PHB accumulation.

Analysis of PHB Film Surfaces by AFM Technique

Morphology and surface roughness of PHB film have been studied by the AFM technique. This experiment is important for surface characterization because the state of surface determines not only mechanism of degradation but the protein adsorption and cell adhesion, which are responsible for polymer biocompatibility and application [38]. As the standard sample we have used the PHB film with relatively low molecular weight. In the film casting procedure the polymer film was adjacent to air. As it is shown in Fig. 10 the surface has a plenty of pores with the length of 150–600 nm. At higher magnification (not presented here) in certain localities the stacks of polymer crystallites with the width of about 100 nm and the length of 500–600 nm can be observed. It can be concluded that the topology characteristic of this biopolymer and its difference in different research is related to the conditions of solvent evaporation during the film casting. During chloroform evaporation the flux forms additional pores, which are fixed as far as the film is solidified and crystallized. The pores on the surface provide fast water diffusion into the bulk of PHB. This finding shows that along with the processes of polymer degradation by microbes the surface hydrolysis can proceed in environment.

Conclusion

The result of this investigation showed that the *A. eutrophus* had the ability to utilize various carbon and nitrogen sources as good substrates for growth as well as PHB production. Of the carbon and nitrogen sources

tested, fructose was the best carbon source with the highest PHB yield of 2.16 g/L and ammonium chloride was selected as the best nitrogen source with PHB production of 0.66 g/L.

Factors like carbon and nitrogen sources and their concentrations have always been of great interest to the researchers in the PHB production for the low-cost media design. It is also known that 30–40% of the production cost of PHB production is the cost of carbon sources. Therefore, it is of great significance to optimize the conditions for cost-efficient PHB production. However, investigations on the impact of carbon and nitrogen supplements had revealed that not just carbon and nitrogen sources would act as enhancer for PHB production. However, role of PHB recovery is also critical at industrial scale. The present study thus indicated that *A. eutrophus* produced high amounts of PHB in minimal medium, which has been modified with certain carbon and nitrogen sources. Further experiments will, however, have to be done using byproducts that are rich in fructose for making the process of production cost-effective.

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