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A Novel Approach to Enhancement of Poly-*B*-Hydroxybutyrate Accumulation *Aulosira Fertilissima* by Mixotrophy And Chemohetertrophy.

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ABSTRACT

Aulisira fertilissima, a unicellular cyanobacterium, produced poly-β-hydroxybutyrate (PHB) up to 5.4% (w/w) dry cells when grown photoautotrophically but 8.9% when grown mixotrophically with 0.2% (w/v) glucose and acetate after 24 days. Gas-exchange limitations under mixotrophy and chemoheterotrophy with 0.2% (w/v) acetate and glucose enhanced the accumulation up to 17–19% (w/w) dry cells, the value almost 4-fold higher with respect to photoautotrophic condition. These results revealed high potential of *Aulisira fertilissima* in accumulating PHB, an appropriate raw material for biodegradable and biocompatible plastic. PHB could be an important material for plastic and pharmaceutical industries.

Keywords: chemoheterotrophy, mixotrophy, *Aulosira fertilissima*, poly-β-hydroxybutyrate

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INTRODUCTION

Infact, Polyhydroxyalkanoates (PHAs) are the polymers of hydroxyalkanoates, which has gained tremendous impetus in the recent years because of its biodegradable and biocompatible nature and can be produced from renewable sources. PHAs are accumulated as a carbon and energy storage material in various microorganisms usually under the condition of limiting nutritional elements such as N, P, S, O, or Mg [1] in the presence of excess carbon [2]. Many of these bacterial species produce the polymer up to 20% of the dry cell weight (dcw) and a few, such as, *Ralstonia eutropha*, now called as *Wautersia eutropha*, is capable of accumulating poly-β-hydroxybutyrate (PHB) up to almost 80% of the dcw [3]. Cyanobacteria, however, are indigenously the sole prokaryotes that accumulate PHB by oxygenic photosynthesis. PHB production using CO_2 as a carbon source by cyanobacteria is available but the contents, in general, are very low and amount only up to 6% (w/w) dry cells [4], with sole exception to *Synechococcus* sp. MA19 where a higher PHB content up to 27% (w/w) dry cells has been reported [5]. Some cyanobacteria can have the ability to produce and accumulate PHB when grown mixotrophically with acetate. The maximum value has been recorded for *Synechocystis* PCC 6803 (15%, w/w dry cells) under nitrogen-limited conditions [6]. Enhanced PHB production by recombinant cyanobacteria has also been reported; the highest accumulation amounts up to 25% (w/w) dry cells in a transformant, of *Synechococcus* PCC 7942 having PHB-synthesis genes from *Ralstonia eutropha* [7] *Aulosira fertilissima*, a N_2 -fixing bacteria is being used as a model cyanobacterium all over the world. Obviously, *Aulosira fertilissima*, has distinguishing feature of spontaneous transformability coupled with short generation time made it one of the most popular organisms for scientific research. Therefore, keeping in view the stimulatory role of nutrient limitation [8], dark incubation [9] and carbon supplementation [10] on PHB accumulation several experiments under similar condition have been designed with innovative prospective in mind under these conditions. A significant increase in accumulation of PHB measured when cynobacterium was cultured in acetate and citrate suplimented medium and reached maximum under deficiency of P in acetate supplemented medium [11]. In the present study variables of cultural and nutritional conditions have been employed to promote high Polyhydroxyalkanoates accumulation. It was found that like chemoheterotrophy and mixotrophy under limitations of gas-exchange give higher accumulation which major influence in higher yield of product.

MATERIALS AND METHODS

Test Organism and Experimental Conditions

Axenic culture of *Aulosira fertilissima* was grown axenically in NO_3 free BG11 medium [12] at 25 ± 1^{o}C, pH 8, under 14 h light (75 µmol photon m^{-2} s^{-1} PAR):10 h dark cycles. Mixotrophic growth conditions were obtained by supplementing different concentrations of glucose, fructose and acetate to the standard mineral medium, without changing the other culture parameters. For chemoheterotrophy, cultures supplemented with different carbon doses were incubated in dark. Gas-exchange limitations in the culture vessels were imposed by wrapping the cotton plugs with aluminum foils followed by tightening with cellophane tapes.

Extraction of PHB

Aulosira cells (dry biomass: 30–40 mg) were centrifuged and suspended in methanol (4^{o}C, overnight) for the removal of pigments. The pellet obtained after centrifugation was dried at 60^{o}C and PHB was extracted in hot chloroform followed by precipitation with cold diethyl ether. The precipitate was centrifuged at 11000 g for 20 min, washed with acetone and dissolved again in hot chloroform [13].

Analytical procedures

Cell dry weight was determined gravimetrically following Rai *et al*., [14]. PHB concentration was determined following the propanolysis method of Riis and Mai , [15] using a GC (Clarus 500; Perkin-Elmer, Shelton, CT, USA) in split mode (1:50, v/v), equipped with Elite-1 dimethylpolysiloxane capillary column (30 m×0.25 mm × 0.25 µm) and flame ionization detector. Benzoic acid was used as the internal standard. Dissolved oxygen (DO) content in the culture vessels was measured using a DO meter (Oxi 330i/SET, WTW, Germany). All experiments were performed in triplicate to check the reproducibility.

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RESULTS AND DISCUSSION

PHB accumulation under mixotrophic conditions, intracellular accumulation of PHB appeared to be different carbon-sources [16, 11]. The stimulatory effect of acetate on PHB accumulation could be due to the direct utilization of acetate for the synthesis of the polyester by means of the usual pathway operating in prokaryotes [17]. Glucose utilization in cyanobacteria, however, occurs via pentose phosphate pathway. Thus, the positive effect of glucose on PHB production could be attributed to the increased supply of the reduced cofactor NADPH [8], which is a prerequisite for the activity of the enzyme acetoacetyl-CoA reductase for conversion of acetoacetyl- CoA to β-hydroxybutyryl-CoA. A similar explanation could also be valid for the increased PHB contents in fructose supplemented cultures. A rise in PHB content up to 8.9% (w/w) dry cells in glucose + acetate supplemented cultures could be explained as the combined effects of the plentily available precursor, i.e. acetate, and the cofactor NADPH. In bacteria, utilization of cyclohexane takes place by oxidation and hydrolysis, followed by β -oxidation and release of acetyl- CoA. The insignificant impact of cyclohexane on the PHB content of *Aulosira fertilissima* points towards non-existence of cyclohexane utilizing pathway in the cyanobacterium under this study, therefore there is an urgent need of an advanced and extensive.

Gas-exchange limitations and PHB accumulation

Gas-exchange limitations under mixotrophy were found to enhance PHB accumulation significantly. One possible reason for this could be nitrogen limitation. Enhanced PHB production in mixotrophically-grown cyanobacteria was observed when nitrate become limited in the medium [6]. During the present study there has been a significant rise in PHB content in 0.2% acetate + glucose grown cultures under limitations of gas exchange was considerably higher, ca. 17-19% (w/w) dry cells as compared to the value reported for Synechocystis PCC 6803, i.e. 15% (w/w) dry cells.

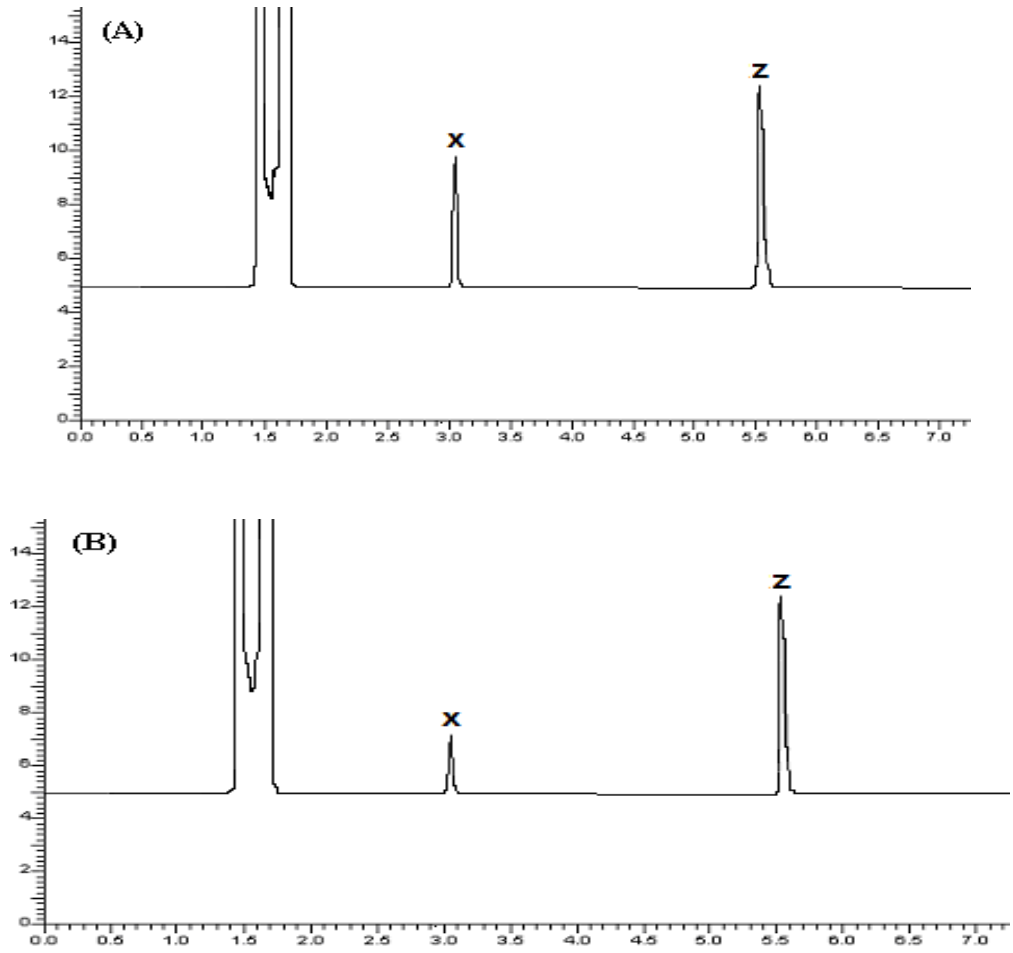

Figure 1: Gas chromatograms of (A) standard PHB and (B) sample.[x: 3-hydroxybutyric acid propyl ester and z: benzoic acid propyl ester (internal standard)].

However, an enhanced PHB accumulation under O_2 limitation was also observed. O_2 limitation results in cascading effect of secondary metabolic events, including cessation of TCA cycle flux and an increase in acetyl-CoA/ CoA ratio (18). As acetyl-CoA is a prerequisite for the activity of ketothiolase, the first enzyme of PHB biosynthetic pathway, an increase in acetyl-CoA/CoA ratio plays an important role in the enhanced PHB accumulation during oxygen limitation. Thus, the observed rise in PHB pool under limitations of gas-exchange could possibly be due to the combined effects of limitations of N_2 and O_2, as in sealed cultures the possibility of limitations of both the gases could not be ruled out.

PHB accumulation under chemoheterotrophy

An increase in PHB accumulation under dark incubation (Table 1) could be ascribed to the limitation of O_2, as photosynthetic O_2 evolution does not take place in the dark. Addition of glucose or acetate at the initiation of dark incubation was found to promote PHB production profoundly. This could partially be related to the increased pool of NADPH under dark in presence of supplemented carbons, as reported for Aphanocapsa 6714 [19]. Moreover, the fact remains that the role of acetate, a precursor of PHB, cannot be denied in the present study.

Table 1: Accumulation of PHB in *Aulosira fertilissima* under chemohetrotrophy after 7 days incubation

Treatment	PHB(% dcw)
Control	5.4 ± 0.40
Dark	3.6 ± 0.69
Dark + G (0.4%)	6.8 ± 0.23
Dark + A (0.4%)	4.9 ± 0.26
Dark + G (0.2%) + A (0.2%)	10.9 ± 1.59
Dark + G (0.4%) + A (0.4%)	6.2 ± 0.69

*Cells were grown for 24 days in L/D cycles followed by chemohetrotrophy.All the values are mean ± SE, n=3. A: acetate, G: glucose

It is obviously concluded that *Aulosira fertilissima* accumulated a considerable amount of PHB under mixotrophy, chemoheterotrophy and mixotrophy under limitations of gas-exchange. PHB content reached up to 17–19% cell dry weight. Some studies proved that product may increase higher amount as 7 fold from *lactobacillus acidophillus* [20] Therefore, the possibility of utilizing cyanobacterial strains in PHB production should not be discarded before assessing their actual potential, and research efforts must be directed to achieve information on the factors that can trigger PHB biosynthesis. Some advance technique may be implemented for improvement in the production and found Optimization of process parameters by response surface methodology resulted into polymer accumulation up to 85% (dcw) at 0.26% citrate, 0.28% acetate, and 5.58 mg L−1 K2HPO4 for an incubation period of 5 days [11].

CONCLUSION

This study indicates increase level of accumulation of poly -*β* -hydroxybuterares after growing *Aulisira fertilissima* mixotrpically. Gas exchange limitations show significant increase in accumulation. This was due to stimulation cause by glucose and acetate which affect cellular biochemical pathway within cynobacterium in addition of limiting in concentration of N_2 and O_2. This is an effort to encourage some new process to enhancing the production of polyhdroxybutarte for its novel applications.

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