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Research Article

Optimization of PHA Production by *Azotobacter fabrum* Isolated from Soil Sample

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ABSTRACT

The inappropriate disposal of synthetic plastics has led to severe environmental health hazards. In order to combat these persistent issues, the synthesis of biodegradable polymers has been gaining interest recently. In the current study, an attempt was done to optimize the production of Polyhydroxyalkaonoates (PHA) by Azotobacter fabrum by adjusting various physicochemical conditions during the growth of the bacterium. The parameters optimized in our study were temperature, pH, aeration conditions, incubation period and carbon to nitrogen ratio. It was observed that A. fabrum produced maximum PHA at pH 7 and temperature 30°C in 48h. The most favoured carbon and nitrogen sources for PHA production were glucose and ammonium nitrate respectively, and the optimum C: N ratio was observed to be 50:1. On optimization approximately 33.02% increase in productivity was observed.

Keywords: *Azotobacter fabrum, Polyhydroxyalkaonoates, Optimization, Biodegradable polymer.*

INTRODUCTION

An important development in an evolving life form is the acquisition of characteristics, which bestow upon the organism the ability to survive the adverse conditions imposed by nutrient starvation. One of the examples is the production of biopolymers by several micro-organisms. Once these characteristics are acquired, the life form then has a more secure basis for further development and diversification. In general, protection against starvation can be achieved by the organism accumulating reserve compounds (eg. biopolymers) during conditions of nutrient sufficiency and then degrading these storage materials during times of starvation, thereby maintaining viability. A wide variety of gram positive as well as gram negative bacteria, capable of biopolymer production, has been reported in literature. However, very few bacteria produce high concentrations of these polymers during their growth under natural environmental conditions. This is because, they are continuously utilized for energy requirements by the bacterial cells, and the rate of consumption may exceed the rate of its production (Silva & Garcia-Cruz, 2010).

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Among the biopolymers, the production of polyhydroxyalkanoates (PHAs) has been widely reported in bacteria. The PHAs are accumulated by different organisms hence they vary in properties and chemical composition due to different monomeric structure. They may exist as either homopolymer or copolymers. They are accumulated in the cytoplasm of bacterial cells as granules under surplus carbon, and limiting conditions of at least one other nutrient (like nitrogen or phosphate), which is essential for growth (Aramvash et al., 2015). The type of PHA produced is dependent on the substrate utilized during the process of biosynthesis, and the properties may differ depending on the monomer composition of the substrate and its molecular weight (Pacheco-Leyva et al., 2016). These polymers have properties similar to synthetic plastics and have gained much attention as future biodegradable plastics (Lee, 1996).

Although several bacteria accumulate PHA intra-cellularly, its industrial production has been possible using few bacteria including *R. eutropha* and recombinant *E. coli*. These organisms, however, have shown limited potential due to factors such as low stability and requirement of expensive carbon sources. Hence it is necessary to explore other microorganisms for production of PHA and other biopolymers. Since nature exhibits diverse climatic and environmental conditions, there is always a scope to discover better producers. In this regard, the potential of indigenous Rhizobacteria isolates like *Azotobacter* sp. has been suggested by several researchers for their capacity of PHA accumulation.

The Azotobacters are obligate aerobic bacteria that can grow on simple mineral salts medium containing basic sugar to produce PHA. The *Azotobacter* sp. carry out a variety of metabolic pathways in order to fix atmospheric nitrogen and they can easily direct excess carbon towards the synthesis of biopolymers such as polysaccharides or polyhydroxyalkanoates. In general, the polysaccharide synthesis is a common feature of *Azotobacter* sp. Another advantage of these isolates is their ability to utilize various toxic and agricultural waste compounds, and produce valuable biopolymers. This process enhances the feasibility of industrial production of PHA, which otherwise, depends on the use of expensive raw materials like glucose and sucrose (Belder, 1993, & Kang et al., 1993). The content of biopolymers in rhizobia is reported to be in the range of 30-65% of dry cell weight (Tombolini & Nuti, 1989). Kim and Chang (1998) reported PHB production in *Azotobacter chroococcum* in presence of starch as carbon source and oxygen limiting conditions. The source of nitrogen used for production of PHB also affects the yield in *Azotobacter* sp. (Kennedy et al., 1982; & Emtiazi et al., 2004).

In this regard, the current study was carried out with an objective to optimize PHA production from *Azotobacter* sp. isolated from soil sample.

MATERIALS AND METHODS

Sample collection and isolation of PHA producer

A PHA producing *Azotobacter* sp. was screened and isolated in a previous study from garden soil sample (Qureshi et al., 2014).

Identification of the potential isolate

The molecular phylogeny of the most promising PHA producing *Azotobacter* sp. was determined by amplifying genomic partial 16srRNA. Based on the similarity in sequences of *Azotobacter* strain with other bacteria, a phylogenetic tree was also obtained to identify the most probable strain. The analysis was carried out at Codon Bioscience Pvt. Ltd, Goa, India.

Extraction of biopolymer using solvent extraction method

The cell biomass was treated with 60 times volume of chloroform and allowed to rest for 4h at 30°C in an extraction funnel. Water was added to keep the film static for 12h. On formation of a clear polymer, it was separated from the solvent mixture and poured in a watch glass for drying, in order to ensure removal of chloroform. The obtained sample

of biopolymer (in powdered form) was dissolved in chloroform and quantified (Ramsay et al., 1994).

Quantification of extracted biopolymer

The biopolymer obtained after solvent extraction process was quantified using Slepecky and Law (1960) method with the help of UV spectrophotometer at 235nm. For this purpose, a standard graph of crotonic acid assay was prepared and the estimation of polymer was done by extrapolating the values obtained for test sample on standard graph. Crotonic acid was used in the assay since, the action of heat causes de-polymerization of PHA into crotonic acid.

The biomass and yield of biopolymer was calculated using the following formula.

$$Biomass = \frac{Weight\ of\ test\ tube\ containing\ dried\ cells}{Weight\ of\ empty\ tube}$$

$$Biopolymer\ yield = \frac{Weight\ of\ dried\ biopolymer}{Dried\ biomass}\ x\ 100$$

Optimization of parameters for biopolymer production

The optimization of PHA biosynthesis was determined in nutrient broth by inoculating 5% culture suspension and incubating at 28°C for 48h under shaker conditions (110rpm). The PHA was extracted using the optimized extraction method and estimated quantitatively by the method described above (Slepecky & Law, 1960). The different physico-chemical parameters were optimized for biopolymer accumulation by altering one factor at a time, keeping the other variables constant, at a specific set of conditions. All experiments were run in triplicates. The varying factors included pH (5.0, 6.0, 7.0, 8.0 and 9.0), temperature (28°C, 37°C, 45°C and 55°C), aeration (static and shaker) condition and time (24h, 48h, 72h, 96h and 120h). For optimization of different parameters, cultures were grown in 50mL nutrient broth and incubated at shaker conditions. In addition to the above parameters, the effect of different carbon and nitrogen sources were studied under previously optimized conditions. The carbon sources used in our study included fructose, sucrose, glucose, mannitol and glycerol. The sources of nitrogen included sodium nitrate, potassium nitrate, ammonium nitrate, ammonium sulphate and urea. Further, the effect of various carbon and nitrogen ratios was also determined on biopolymer accumulation (Bonartseva et al., 2003; Bormann et al., 1998; & Pal et al., 1998).

RESULTS

Identification of the potential isolate

The PHA producing isolate was identified as *Azotobacter fabrum* on 16srRNA analysis and observations from the phylogenetic tree (Figure 1).

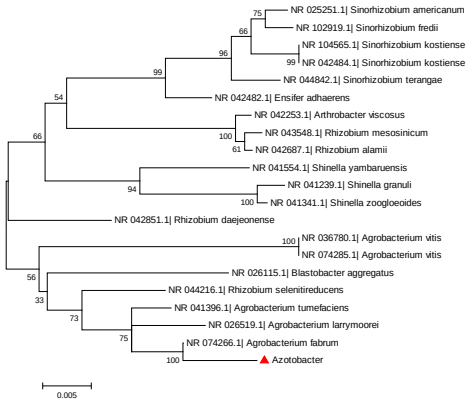

Figure 1: Phylogenetic tree for bacteria (*Azotobacter*) using partial 16S rRNA gene sequence

Extraction and quantification of biopolymer

Azotobacter fabrum was grown in nutrient broth and the biopolymer was extracted using solvent extraction method. For this purpose, the biomass obtained was calculated after centrifugation and drying of the cells. The biopolymer yield was calculated from the standard graph of crotonic acid assay. The biomass, biopolymer concentration and yield of polymer was calculated as 1.063 µg/mL, 0.312 µg/mL and 29.41% respectively.

Optimization of parameters for biopolymer production

The results for optimization of physicochemical parameters for accumulation of biopolymer by *Azotobacter fabrum* are represented below. The optimum conditions favouring biopolymer production were obtained under shaker conditions (Figure 2) of growth in 48 h (Figure 3). The physicochemical conditions like pH 7 (Figure 4) and temperature 30°C (Figure 5) supported the accumulation of PHA in our study. The most favoured carbon (Figure 6) and nitrogen sources (Figure 7) for biopolymer production were glucose and ammonium nitrate respectively, and the optimum C: N ratio was observed to be 50:1 (Figure 8) for maximum biopolymer production. Thus our studies indicated production of biopolymer in stationary phase and nitrogen limiting conditions by *Azotobacter fabrum*. Under optimized conditions, the PHA yield increased from initial 29.41% to 43.91%.

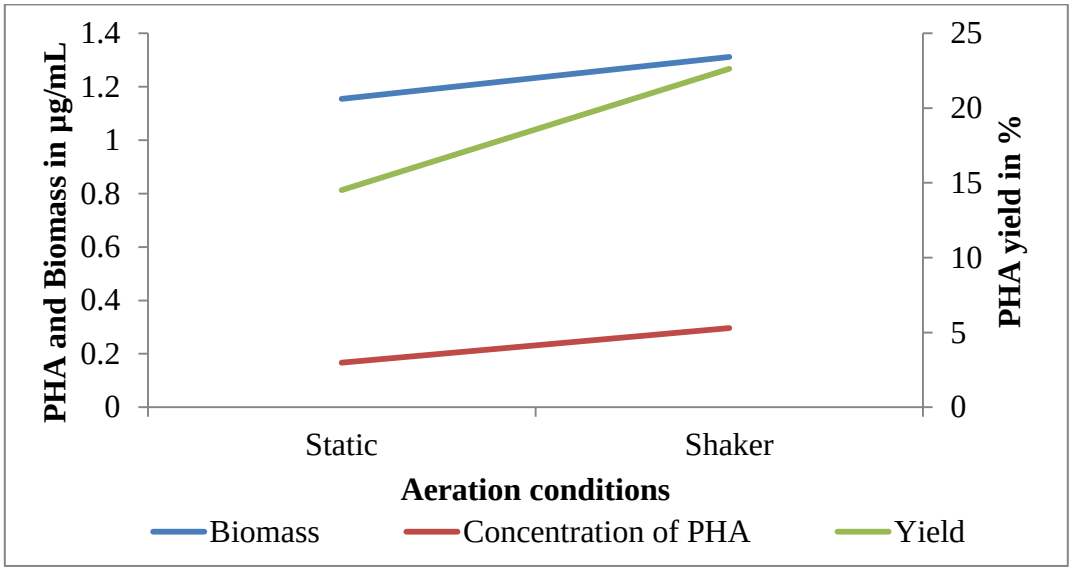

Figure 2: Optimization of aeration condition for biopolymer production from *Azotobacter fabrum*

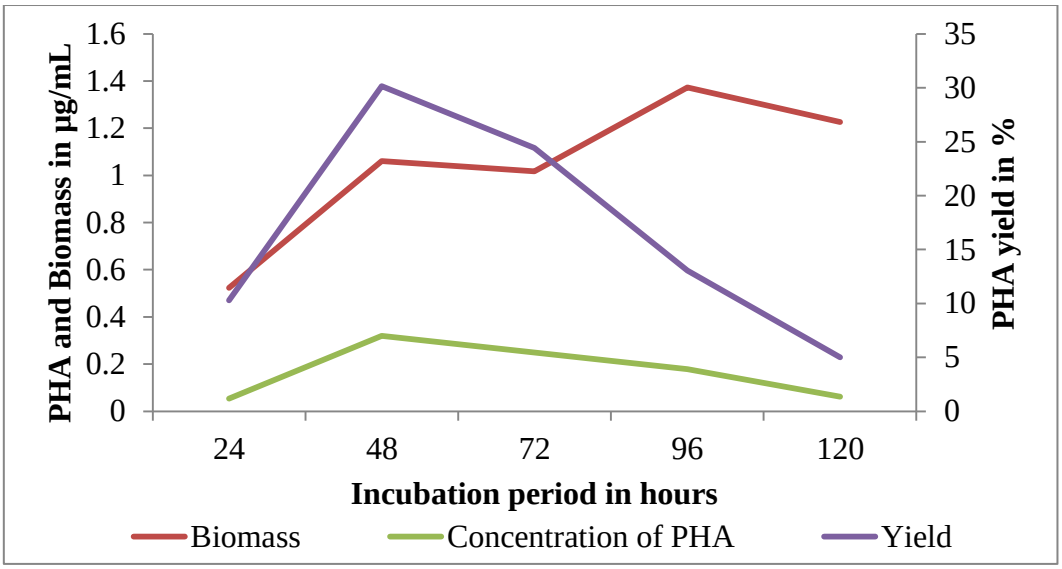

Figure 3: Optimization of Incubation period for biopolymer production from *Azotobacter fabrum*

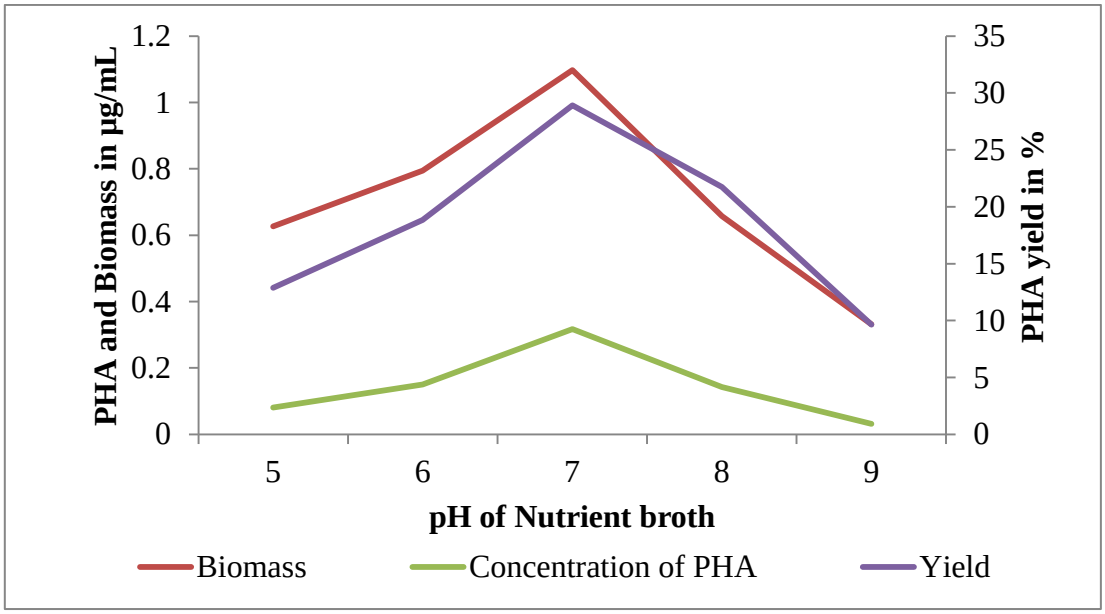

Figure 4: Optimization of pH for biopolymer production from *Azotobacter fabrum*

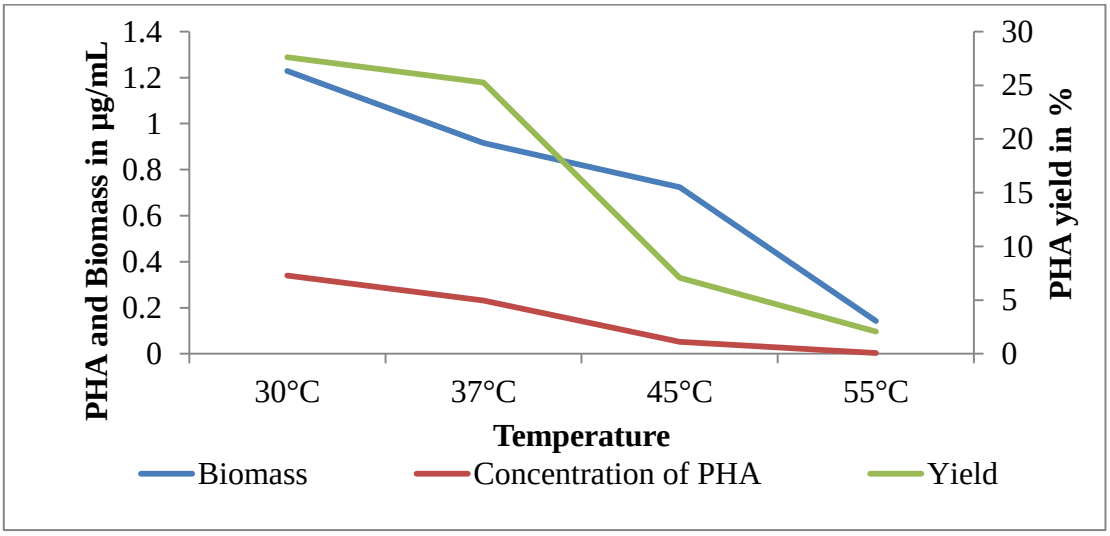

Figure 5: Optimization of Temperature for biopolymer production from *Azotobacter fabrum*

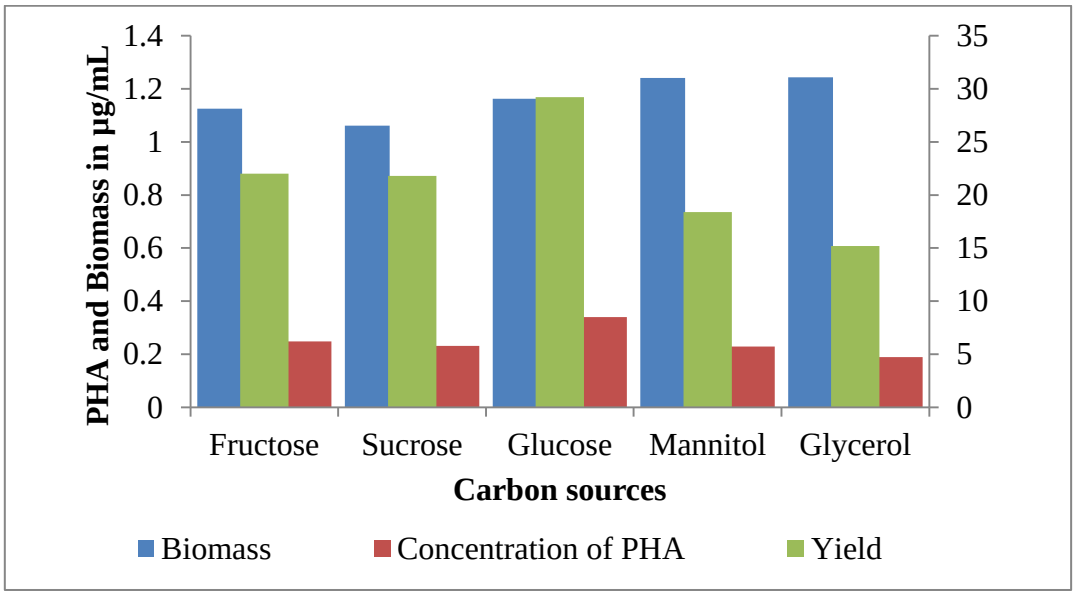

Figure 6: Optimization of Carbon sources for biopolymer production from *Azotobacter fabrum*

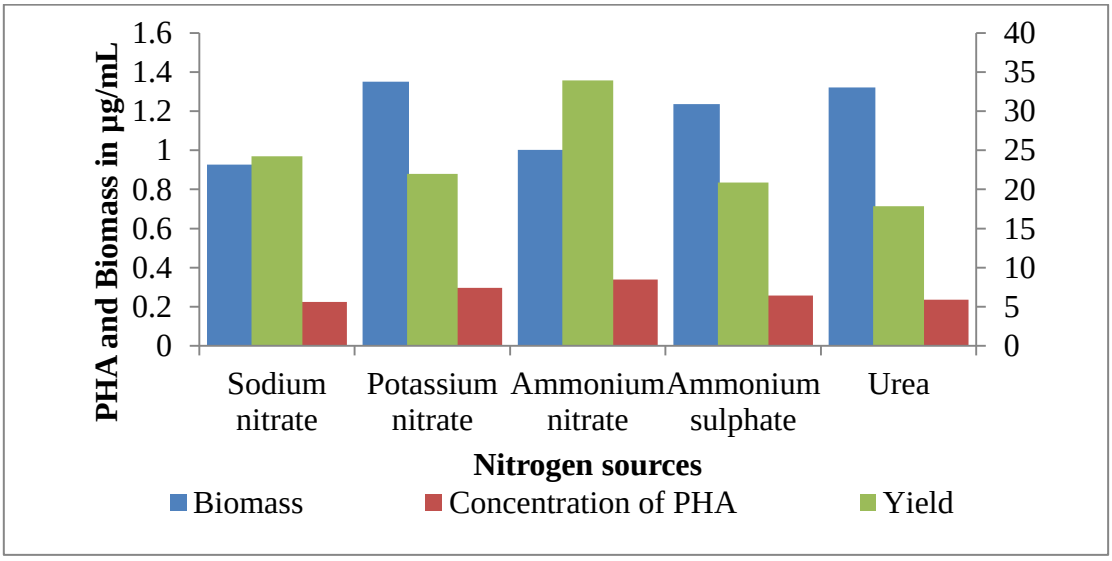

Figure 7: Optimization of Nitrogen sources for biopolymer production from *Azotobacter fabrum*

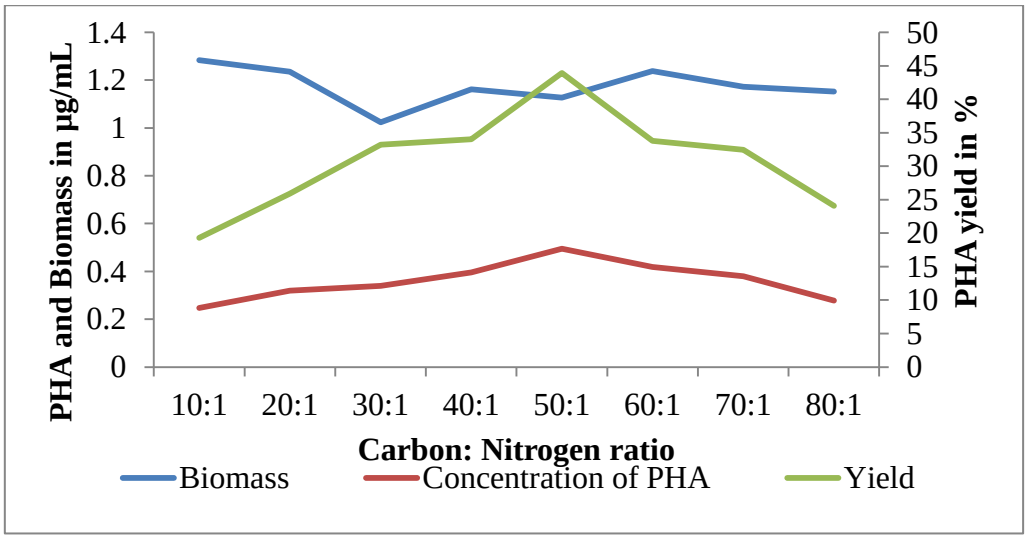

Figure 8: Optimization of Carbon: Nitrogen ratio for biopolymer production from *Azotobacter fabrum*

DISCUSSION

Every activity, occurring in nature, is a part of complex interconnected biochemical cycle that solely serves the purpose of maintenance of sustainability of natural flora. The production of biopolymer is a mechanism developed by micro-organism, during the evolution, to reserve excess carbon sources and utilize them during nutrient limiting conditions. A vast diversity of micro-organisms is known that are capable of accumulating a variety of biopolymers intra-cellularly. Understanding of these compounds has revealed the industrial importance of biopolymers, especially PHA, as a substitute for synthetic plastics.

Generally, under carbon surplus and nitrogen limiting conditions bacteria follows the 'overflow metabolism'. During this phenomenon, the unbalanced supply of nutrients causes cessation of complex metabolic pathways occurring in bacteria and directs the flow of carbon uni-directionally towards PHA synthesis (Nubia et al., 2007). Commonly, the limitation of nitrogen, magnesium, oxygen, phosphate and potassium stimulates PHA accumulation. However, limitations of iron and sulfur have also been reported to initiate PHA synthesis (Babel et al., 2001). Similar to our study, rhizosphere soil has been used for isolation of PHA producers by few authors (Kumbhakar et al., 2012; & Naheed et al., 2011).

Azotobacter sp. was selected for the current study because of its common

occurrence in soil. For any industrial process, the ready availability of raw material is a crucial requirement. Not only are *Azotobacter* sp. easy to isolate but also they are easier to maintain under laboratory conditions. Moreover, unlike most bacteria that produce PHA in the stationary phase of growth, *Azotobacter* sp. are capable of PHA production during active growth. This factor can significantly improve the product yield on optimization. In nitrogen fixers like *Azotobacter beijerinckii*, *Azotobacter insignia* and *Rhizobium* ORS571, PHA synthesis within the nodule gets induced under conditions of stress like low oxygen state or diminished redox potential (Saharan & Badoni, 2007; Senior et al., 1972; Stam et al., 1986; & Stockdale et al., 1968).

The production of PHAs is dependent on various factors like type of the organism, cell density, the constituents present in the nutrient medium, and physico-chemical parameters like pH, temperature, aeration and incubation period. Thus, the optimization of these factors is a crucial step to maximize the yield of PHA from micro-organisms. For a medium to be selected as an optimal production medium, it should enable cell biomass as well as PHA accumulation. The pH and temperature has an impact on metabolite production and microbial growth. An increase in pH beyond optimum may cause changes in solubility of media components and cell permeability (Umesh et al., 2017). It may also lead to degradation of biopolymers resulting in PHA depletion at the same rate as its production (Nakata, 1963). Temperature affects the growth of bacteria by regulating the metabolic activities, biochemical composition and enzymatic functions of a cell and hence affects PHA yield (Inagraham, 1962). Elevated temperatures may cause thermal inactivation of the enzymes responsible for biosynthesis of PHA (Tajima et al., 2003). The size of the inoculum also influences the length of the lag phase of the culture which successively affects the growth, the efficiency of biopolymer production and the incubation time. Hence, the optimum PHA production can also be achieved using a two stage strategy, whereby the cells are grown in a suitable medium initially to increase biomass followed by exposing them to stress conditions that ensures PHA accumulation (Madison & Huisman, 1999).

Oxygen is essential for sustenance of the reducing power. Minor difference in oxygen availability affects the tricarboxylic acid cycle (TCA cycle) leading to significant changes in the metabolite distribution, including biopolymer accumulation, in various micro-organisms (Almeida et al., 2010). The high cell mass leading to oxygen depletion during growth phase hinders the TCA cycle resulting in accumulation of NAD (P) H that inhibits citrate synthase. The accumulated cofactor is then used by various organisms for PHA synthesis (Quillaguaman et al., 2006). Almeida et al. (2010) discovered that substantial variations in the metabolite distribution of various organisms can be observed due to small variations in oxygen availability. Lafferty et al. (1988) detected stimulation of PHA accumulation under oxygen limited conditions in *Ralstonia eutropha* and *Azotobacter beijerinckii*. Research has proved that conserving oxygen is essential for sustaining the reducing power and excessive aeration reduces PHB yield (Third et al., 2003; & Salehizadeh & Van Loosdrecht, 2004). PHA accumulation was influenced by aeration, accumulation was the highest under shaker conditions in a flask containing less medium whereas a decrease was reported as the volume of medium increased (Kato et al., 1992). Suzuki et al. (1986) reported that in *Pseudomonas* sp. K, the rate of growth and PHA synthesis decreased with limitation in concentration of dissolved oxygen. Research has shown that with increase in agitation rate PHA yield reduced which may be due to the stress created by oxygen limitation at lower rpm, leading to increase in PHA accumulation (Wei et al., 2011).

In the current study, 29.41% PHA yield was obtained from *Azotobacter fabrum* under random environmental conditions. On optimization, it increased up to 43.91% under

optimized conditions of growth. This is approximately 33.02% increase in productivity. These optimized growth conditions for PHA production by *Azotobacter fabrum* was pH 7, temperature 30°C, and shaker conditions. The presence of glucose and ammonium nitrate in the ratio 50:1 was the optimum nutritional parameter noted in our study.

CONCLUSION

The polyhydroxyalkanoates are a biodegradable energy reserve material of micro-organisms. Most interestingly, they can be exploited as a substitute to petroleum-derived plastics. The fundamental studies leading to industrial application of such products are critically essential for a much needed sustainable development processes. A simple yet efficient technology is possible with the help of microbiology to develop an integrated system for continuous production of valuable products like bioplastics. The minute cell factories are capable of endless production of high-valued industrially relevant metabolic products, provided they obtain the necessary growth and nutritional requirements. We have definitely come a long way, through biochemical insights, in our understanding of PHA biosynthesis, triggers and inhibitions. With the help of similar studies we can soon establish an industrial process contributing positively to overcome key elements that present themselves as obstacles for current implementation of bioplastic applications. Most importantly, the relevant questions on cost of production and yield of PHA can be hopefully answered in near future. In addition, such studies open a whole new dimension for understanding of various interlinked metabolic processes that connect the ecological prospects like diversity and evolution. Moreover, successful implementation of microbial based plastics will help in overcoming environmental pollution.

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