SYNTHESIS OF EXTRACELLULAR AND INTRACELLULAR POLYMERS IN ISOLATES OF *AZOTOBACTER* SP

A.A.Haleem Khan[1], Naseem[2], B. Vidya Vardhini[3]

[1]*Dept. of Botany, Telangana University, Dichpally, Nizamabad-503322*
[2]*Dept. of Pharmaceutical Chemistry, Telangana University, Dichpally, Nizamabad-503322*
aahaleemkhan@gmail.com

Abstract

Soil is principal source of diverse group of microorganisms. The bacterial genera-*Azotobacter* is known asymbiotic diazotroph for long time and used as biofertilizer with multipurpose activities that improve plant productivity by maintaining sustainable soil health. *Azotobacter* species (*A. beijerinckii, A. chroococcum, A. vinelandii*) synthesize extracellular (alginate) and intracellular polymer (Poly-β-hydroxybutyrate). Alginates are linear copolymers of β-D-mannuronic acid and α-L-guluronic acid. Poly-β-hydroxybutyrate is lipid inclusion body, common in wide range of bacteria. Both the polymers are commercial components with high applied value in different fields. *Azotobacter* species are used as bio-inoculants in various cultivable crops to improve yield as they fix atmospheric nitrogen asymbiotically. In the present study we have screened the rhizospheric samples of cultivable crop plants like tomato, chili, rice, sugarcane and maize for *Azotobacter* species. The bacterial isolates were identified based on morphological and biochemical methods. Whey, wheat bran and molasses were used as cheap substrates for production of polymers. The substrates used in our study are inexpensive and could reduce the production cost of these polymers.

Keywords: *Alginates, Poly-β-hydroxybutyrate (PHB), whey, wheat bran, molasses*

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INTRODUCTION

Bacteria are known to synthesize a wide range of biopolymers that could contribute diverse biological functions and numerous industrial and medical applications [5]. Current research in the field of bacterial biopolymers has developed new avenues for the engineering of bacteria and produce tailor made biopolymers [6]. Louis Pasteur in mid-19th century was first to discover bacterial polymer dextran as a product in wine. Bacteria utilize different carbon sources as substrate in synthesis of different polymers [7]. Extracellular polymers are synthesized in vast compared to intracellular polymers by bacteria [8].

Azotobacter sp. is free living, nitrogen fixing, obligate aerobe common in rhizospheric soil from tropical and temperate regions [9]. These species are isolated with simple, oxidizable substrates i.e. sugars as carbon and energy sources, it also oxidize many aromatic compounds [13]. Alginate is a polysaccharide that belong to the family of linear, non-repeating copolymers, with β-D-mannuronic acid and α-L-guluronic acid linked by β-1,4-glycosidic bonds [15]. Cell walls of marine brown algae such as *Laminaria* and *Macrocystis* are sources of commercial alginates and bacterial genera like *Pseudomonas* and *Azotobacter* also produce alginate [17]. This polymer has wide range of application, stabilizing, thickening, gel or film forming agents in industrial fields (food, textile, pharmaceutical) and source of soluble fibre in medical products. Alginate is important constituent of cysts of *Azotobacter* sp. under unfavorable conditions.

Poly-β-hydroxybutyrate (PHB) is lipid inclusion body, common in wide range of bacteria. This polymer is a biopolyester used as biodegradable plastic. Under stress conditions such as nutrient limitation PHB serves as carbon and energy sources. Several million tons of plastic waste around the globe is important issue that depletes the ecosystems. These conventional synthetic plastics are non degradable and resistant to microbial attack as they lack hydrolyzing enzymes. PHB is accumulated in bacteria during stationary phase of growth [19].

In the present study we have screened the rhizospheric samples of cultivable crop plants like tomato, chili, rice, sugarcane and maize for *Azotobacter* species. The bacterial isolates were identified based on morphological and biochemical methods. Whey, wheat bran and molasses were used as cheap substrates for production of polymers.

Materials and Methods

Bacterial culture

The rhizospheric soil samples of cultivable crop plants like tomato, chili, rice, sugarcane and maize were used for isolation of *Azotobacter* species on Ashby's mannitol agar. The bacterial isolates were identified as *Azotobacter chroococcum* and *Azotobacter vinelandii* based on morphological and biochemical methods. The isolates were maintained mannitol broth at 37°C for 24 h on rotary shaker at 160 rpm.

Production of Alginate

Burk's medium was prepared and 1ml of cultures (*A. chroococcum* and *A. vinelandii*) were inoculated in two separate conical flasks and incubated at 37°C for 48 h on rotary shaker at 300 rpm. After completion of incubation the cells were checked for alginate by violamine stain.
The isolates of *A. chroococcum* and *A. vinelandii* were inoculated in Burk's medium with 1% whey, wheat bran and molasses in separate flasks and incubated at 37°C for 72 h. The biomass was harvested by centrifugation and alginate was extracted. The alginate extract was checked by carbazole test.

Production of Poly-β-hydroxybutyrate (PHB)

Burk's medium was prepared and 1ml of cultures (*A. chroococcum* and *A. vinelandii*) were inoculated in two separate conical flasks and incubated at 37°C for 48 h on rotary shaker at 300 rpm. After completion of incubation the cells were checked for PHB by Sudan black stain.
The isolates of *A. chroococcum* and *A. vinelandii* were inoculated in Burk's medium with 1% whey, wheat bran and molasses in separate flasks and incubated at 37°C for 72 h. The biomass was harvested by centrifugation and PHB was extracted.

Results and Discussion

The bacterial colonies formed were small, transparent, circular, flat, slimy with regular border, pigments (yellow-green and brown) and the pigments difference made to divide isolates in two types of species. Morphological and biochemical properties were gram negative, short rods with round ends, motile, the tests such as catalase, oxidase and nitrate reduction were positive. Sugar fermentation tests difference classified the isolates as *A. chroococcum* and *A. vinelandii.*

Isolates of *A. chroococcum* and *A. vinelandii* were selected for alginate synthesis based on violamine staining. *A. chroococcum* grown in medium inoculated with whey, wheat bran and molasses showed biomass (g) 2.5, 3.9 and 3.1, the dry weight (g) was 2, 2.6 and 2.3; alginate yield (%) was 40, 42, and 41; PHB yield (%) was 50, 58, and 55. *A. vinelandii* grown in medium inoculated with whey, wheat bran and molasses showed biomass (g) 3.5, 3.3 and 2.8, the dry weight (g) was 2.8, 2.4 and 2.1; alginate yield (%) was 44, 40, and 39; PHB yield (%) was 60, 62, and 65.

In several studies on *A. vinelandii* under oxygen-limited and non-limited conditions revealed impact on polymer concentration and its composition i.e. molecular weight. Under the oxygen-limited conditions molecular weight of alginate was increased specifically at oxygen concentration near zero. The dissolved oxygen tension and oxygen transfer rate influences on the polymerization and degradation of alginate by *A. vinelandii* [4, 7, 12]. The study on wheat plants cultivated in soils contaminated with heavy metals cadmium (Cd) and chromium (Cr) under pot culture experiments, the isolated strain of *Azotobacter* in free cells form and immobilized cells form showed production of extracellular polymeric substances that conferred heavy metal resistance [11]. The growth studies of *A. vinelandii* in a phosphate and nitrogen-rich medium with glucose influenced the secretion of alginic acid and the accumulation of poly-β-hydroxybutyrate [1]. The mutant strain for *ptsN* gene of *A. vinelandii* under low aeration conditions increased the two fold increase in biosynthesis of poly-β-hydroxybutyrate with high molecular mass [14]. The cultivation of *A. vinelandii* on crude glycerol improved the gelling capacity of alginate suitable to form stable hydrogel for wound healing applications [10].

A poly (3-hydroxybutyrateco-3-hydroxyvalerate) and novel terpolymer, poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-poly(ethylene glycol) was biosynthesized from *A. chroococcum* by using sucrose as carbon source along with valeric acid and poly(ethylene glycol) 300. These polymers were biocompatible and biodegradable used in biomedical products [2, 3]. Three different polymers such as carrageenan, sodium alginate and HPMC were proved to preserve *A. chroococcum*, the results showed that the storage time, temperature and protective agent influenced viability and degradation rates. Carrageenan preserved the test bacterium for ~900 days at 4°C, this showed suitability for preservation and storage conditions [18].

CONCLUSION

A. chroococcum grown in medium inoculated with whey, wheat bran and molasses showed biomass (g) 2.5, 3.9 and 3.1, the dry weight (g) was 2, 2.6 and 2.3; alginate yield (%) was 40, 42, and 41; PHB yield (%) was 50, 58, and 55. *A. vinelandii* grown in medium inoculated with whey, wheat bran and molasses showed biomass (g) 3.5, 3.3 and 2.8, the dry weight (g) was 2.8, 2.4 and 2.1; alginate yield (%) was 44, 40, and 39; PHB yield (%) was 60, 62, and 65. Whey, wheat bran and molasses were used as cheap substrates for production of polymers. The substrates used in our study are inexpensive and could reduce the production cost of these polymers. The soil isolates of *A. chroococcum* was cultured under various carbon sources like glucose, fructose and sucrose along with ammonium sulfate influenced the coproduction of poly-β-hydroxybutyrate and exopolysaccharides [16].

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