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Effect of Nitrogen Sources and Incubation Times on Poly-beta-hydroxybutyrate (PHB) Synthesis by *Azotobacter vinelandii* Isolated from Soils of Guilan Province (North of Iran)

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ABSTRACT

Plastics have resistance to biological breakdown that result in its accumulation in the environment.This emphasizes the need to search for biodegradable polymer, which is easily disposable and degradable. Bacteria synthesize and accumulate polyhydroxy alkanote as nitrogen source and under limiting conditions of nutrients**.** The purpose of this study was to optimization of food ingredient and culture conditions to improve poly-β- hydroxybutyrate produced by *Azotobacter vinelandii* isolated from Deylaman's forests (North of Iran).At first, using one factor at time, effect of different nitrogen sources, as well as incubation time of production was evaluated. Then, optimization in the best nitrogen source was performed. The effect of different nitrogen sources such as bactopepton, casein, yeast extract and ammonium sulphate were investigated in the production of poly-β-hydroxybutyrateby bacterium. Results showed that among nitrogen sources, ammonium sulphate was the best known as nitrogen source. The best time for poly-ß-hydroxybutyrate production by ammonium sulphate was obtained in 48 hours. In general, between concentrations of (1 - 8%) of nitrogen source, the concentration of 6% resulted in highest production. Obtained results suggested that most appropriate carbon source concentration in production of poly-ß-hydroxybutyrate, was not necessarily the highest concentration, but also is the highest concentration that led to inhibition of bacterial growth.

Key words: *Azotobacter vinelandii*, Poly-ß-hydroxybutyrate, Thermoplastic, Optimization, Deylaman's forests.

INTRODUCTION

The development of biodegradable plastics is one of possible solutions for this problem. Poly (hydroxybutyrate-*co*-valerate) has been recognized as a potential environment-friendly substitute for traditional plastics [12]. It is produced via bacterial fermentation and can be easily degraded to water and carbon dioxide under different environmental conditions. Poly-ß-hydroxybutyrateis a natural, biodegradable polymer accumulated in the form of intracellular granules by a large variety of bacterium [5]. The use of synthetic polymers has increased in many different industrials sections, mainly in the package industry, due the high strength, lightweight, low cost, easy processibility, and good water-barrier properties. However, the large volume of plastics materials produced and their fast discard in landfills have caused many problems in the environment due their no-biodegradability. However, high cost, slow crystallization rate, relative difficulty in processing and high degree of crystalline is drawbacks for wide poly (hydroxybutyrate-*co*-valerate) use [2,3, and 12]. The addition of filler frequently contributes to obtain a more competitive price and a general improvement in the load-bearing capability [2]. In this way, lignins have been incorporate into various polymeric materials as low cost filler. Enormous amounts of industrial lignin and derivates are produced as byproducts of papermaking [6]. Poly-ß-hydroxybutyrate is an aliphatic homopolymer of poly-ß-hydroxybutyrate acid with a melting point of 179°C and is highly crystalline (80%). It can be degraded at the temperature above its melting point. Poly-β-hydroxybutyrate has some properties similar to polypropylene with three unique features: thermoplastic processability, 100% resistance to water, and 100% biodegradability [4].

MATERIAL AND METHOD

Soil sampling: Soil samples were collected in different areas of deylaman's forest (North of Iran). Samples were withdrawn at a depth of 10–15 cm below the surface, collected into sterile vials, and stored at field moisture content at 4 °C. Soil pH was measured, analyzing samples thoroughly suspended in distilled water 1:1 (v/v).

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Isolation: Three different isolation strategies were used: (a) streaking of serial soil dilutions on plates containing Burk Nfree medium; (b) enrichment in Winogradsky solution for 7–14 days followed by streaking onto Burk-agar; (c) Rennie medium. Methods realized as follows: about 30–50gram of each soil sample were accurately mixed with 20% (v/w) of sterile water and 0.5g of pyruvate sodium. The soil paste, prepared in a porcelain mortar, was transferred and pressed inside a petri dish with a sterile spatula to obtain a smooth and leveled surface. After 3–7 days incubation at 30 °C, the soil paste plates presenting growth of *Azotobacter* were revealed by the appearance of slimy, glistening colonies, turning brown with aging if produced by the species *Azotobacter*.

Screening of isolates: To differentiate isolates between Gram-positive and Gram-negative, the KOH-test described was used. Cultures giving uncertain results were subjected to Gram staining. Isolates were then screened for growth ability, colony morphology, pigments production, H_2S production, motility, sugars fermentation, catalase test, urease production, growth in various pH and acidification activity on N-free LG medium containing sucrose as sole carbon source and blue of bromothymole as pH indicator. Production of acid, indicated by a change in color of the medium from blue or dark green to a definite yellow, was checked and recorded after 2, 5, or 7 days incubation, as well as pigments production and colony morphology.So; this bacterium can produce poly-ß-hydroxybutyrate granules.

Production of poly-β-hydroxybutyrate

Growth condition: The bacterium used in the experiments was identified*Azotobacter vinelandii***.** This bacterium was growth on Burk and Rennie medium (sucrose 20g/l, K_2HPo_4 0.08g/l, KH_2Po_4 0.2g/l, $MgS0_4$, $7H_2O$ 0.4g/l, $CaCl_2$ 0.3g/l, Na_2MoO_4, 2H2O 0.1g/l, $FeSo_4$, $7H_2O$ 0.7g/l, pH was adjusted to 7 using 1M NaOH) and incubated at 30 °C for 24-48 hours.

Quantification of culture condition: In this studyforquantification, was used from *Azotobacter* Mannitol Broth medium that containing (Dipotasium phosphate 0.1g/l, Magnesium sulphate 0.02g/l, sodium chloride 0.02g/l, ferrous sulphate 0.01 g/l pH was adjusted to 7.3 using 1 M NaOH).

Effect of different nitrogen sources on poly-β-hydroxybutyrate production: The selected bacterium isolate was grown in 250 ml Erlenmeyercontaining 100 ml *Azotobacter* Mannitol Broth with different nitrogen sources (bactopepton, casein, yeast extract, and ammonium sulphate) at 2 per cent level. The Erlenmeyers were incubated at 30°C on a rotary shaker (230rpm) for 24-72 hours and for studying of time course in this concentration, done sampling in 24-72 hours.

Determination ofCell Dry Weight: Firstly, the samples werecentrifuged in 3000rpm for 2 hours. Then supernatant was thrown and remainder sediment in tube that was biomass for drying was place into oven. After 72 hours, the sampleswere weighing and sediment's weight decrease from tubes weight that calculated before sampling. The remainder number was Cell Dry Weight.

Determination of poly-β-hydroxybutyrate: Determination of the amount of poly-ß-hydroxybutyratewas performed chemically. Bacterium was grown on *Azotobacter* Mannitol Broth medium at 30ºC for 48 hours on a shaker 230rpm.Then, the sampleswere centrifuged for 2 hours in 3000rpm and was added 4 per cent sodium hypochlorite to sediments and incubated at 60°C for 1 hour, then was resuspended in equal volume of ethanol 95% and acetone and was added 1ml chloroform, so the samples was place into oven at 40°C for 30 min. Finally, the polymer granules were dissolving in hot chloroform. At the end, was added 10ml hot H_2So_4 and was placed in a water bath 100°C for 10 min. The addition of sulfuric acid converts the polymer into crotonic acid. The solution was cooled and the absorbance read at 235nm against a sulfuric acid blank. By referring to the standard curve, the quantity of poly-ß-hydroxybutyrate produced was determined.

RESULT AND DISCUSSION

The selected bacterium isolate, could ability to use ammonium sulphateas fermentation substrate. *Azotobacter vinelandii* was produced colonies that were mucoid, ropy and capsule positive. The bacterium was Gram-negative rods with rounded ends, formed coccoid cells, even at a very early stage. These coccoid cells showed high content of PHAs granules. Capsule material was observed when *Azotobacter vinelandii* was grown on *Azotobacter* Mannitol Agar. Production of poly-β-hydroxybutyrate was confirmed by chemical extraction, obtaining significant amounts of crystalline, thermoplastic biopolymer. Poly-β-hydroxybutyrates are a class of microbiologically produced polymers that have potential applications as conventional plastics and as thermoplastic elastomers. This biopolymer exhibits material properties similar to polypropylene. Several works were reported on production of poly-β-hydroxybutyrate using easily available and cheap raw material [1] and optimizing culture conditions for the productions of poly-β-hydroxybutyrate with high concentration and

productivity [7]. The accumulation of the poly-β-hydroxybutyrate (dry weight of the cell and biomass) varies with the type of different nitrogen sources (figure1, 2). Maximum poly-β-hydroxybutyrate level was observed in 48 hours culture (figure3). The yield of poly-ß-hydroxybutyrate in *Azotobacter vinelandii* varies with the type of different nitrogen sources in the media. Highest poly-ß-hydroxybutyrate accumulation in *Azotobacter vinelandii* was found with ammonium sulphate (as nitrogen source) (figure4), when ammonium sulphate was used as the nitrogen sources and the best percentage of ammonium sulphate was 6% (w/w) (figure5). Almost similar trend poly-ß-hydroxybutyrate accumulation was observed when casein, yeast extract and bactopepton were used as nitrogen source in the media. General, the poly-ß-hydroxybutyrate accumulation rates were more or less similar in presence of the different nitrogen in the medium irrespective of the incubation period. Plastics have become an important part of modern life and are used in different sectors of operations like packaging, building materials, consumer products and many more. Each year, about 100 million tones of plastics are produced worldwide. Demand for plastics in India reached about 4.3 million tons in 2001-2002 and is expected to increase to about 8 million tons in 2006-7. Currently, however, the per capita consumption of plastics in India is only about 2kg compared to 30-40kg in developed countries [6]. The present market, in India, is about Rs. 25,000 cores. In this study, using of one factor at- a- time method, effect of different nitrogen sources also, incubation period and optimum source percent were investigated. Then optimization of best nitrogen source was performed. Results were analyzed using variance analysis, ANOVA (SPSS). Effect of different nitrogen sources such as bactopepton, casein, yeast extract and ammonium sulphate was studied in the production of poly-ß-hydroxybutyrate by bacterium and after obtaining the best sources, it values was optimized [8] and observed that *Azotobacter vinelandii* was not fit for commercial production because it produced polyhydroxyalkanoate with low yield and formed capsules. Strain UWD of this organism, however, was not of interest because it was a capsule-negative mutant and produced polyhydroxyalkanoate content of approximately 70-80%. In this study isolated *Azotobacter vinelandii* produced poly-ß-hydroxybutyrate by ammonium sulphate in *Azotobacter* Mannitol Broth medium [9]. Isolated aerobic free living nitrogen fixing bacterium from natural environments. Systematic screening of these isolates has indicated that nearly 70 per cent of isolates of genus *Azotobacter* were capable of accumulating poly-ß-hydroxybutyrate. The poly-ß-hydroxybutyrate contents of majority of the strains ranged from 25-47 per cent of cell dry weight, while only 7 isolates accumulated poly-ß-hydroxybutyrate accounting to more than 50 per cent of their cell dry weight. One of the promising strains of *Azotobacter chroococcum* has been shown to accumulate the polymer accounting nearly to 70 per cent of cell dry weight, when grown under optimized conditions [10] tested 37 isolates and mutants of *Azotobacter chroococcum* for poly-ß-hydroxybutyrate production using Sudan black B staining method. With 2 per cent glucose and 15 mom/l ammonium acetate, poly-ß-hydroxybutyrate production was found to be maximum at 36 and 48 hours of growth under submerged cultivation and under stationary cultivation respectively. They also observed that poly-ß-hydroxybutyrate production was higher on sucrose and commercial sugar as compared to glucose and mannitol, like our study. Among inorganic nitrogen sources, they found ammonium acetate (15mMol/l) to be the best for poly-ß-hydroxybutyrate production [11]. Reported that poly (3-hydroxybutyric acid) and copolymer of poly (3-hydroxybutyrate-Co-3-hydroxyvalerate) [P (3HB-Co-3HV)] were accumulated by numerous bacteria as energy reserve material under conditions of unbalanced growth in the presence of excess carbon source. They also reported that *A. chroococcum* MAL-201 (MTCC 3853) was founded to accumulate poly-3-hydroxybutyrateaccounting 68.4% of cell dry weight from glucose (2% w/w) in modified Norris nitrogen free medium. It produced co-polymer poly (3hydroxybutyrate-co-3hydroxyvalerate when propionate and valerate were used as sole source of carbon at low concentration (0.01-0.1% w/w). The polyhydroxyalkanoate content of cell was less than 10 per cent of cell dry weight but the hydroxyvalerate content of the polymer showed wide range of variability ranging from 5.94 to 3.65 mole per cent. Supplementation of glucose with propionate and valerate as precursors at mid log phase of growth, increased the total polyhydroxyalkanoate content up to 68.9 per cent of cell dry weight with 19.31 moll per cent hydroxyvalerate. In studying the relationship between the production of poly-β-hydroxybutyrate and the optimal time to produce, in all cases the reduced production rate, poly-β-hydroxybutyrateproduction time after optimization were observed. This means that work in all cases; we have always been a reduction, which observed in the amount of poly-β-hydroxybutyrate in the logarithmic growth or stationary phase. Because these cases can be sought in the metabolic process of poly-β-hydroxybutyrate, namely the bacterium are encountered to nitrogen deficiency when the amount of carbon sources was increased, surplus carbon sources stored as poly-β-hydroxybutyrate polymer and energy in their cytoplasm as a carbon source. In fact, it made saved as metabolic energy when faced with a shortage of energy and carbon until use this source.

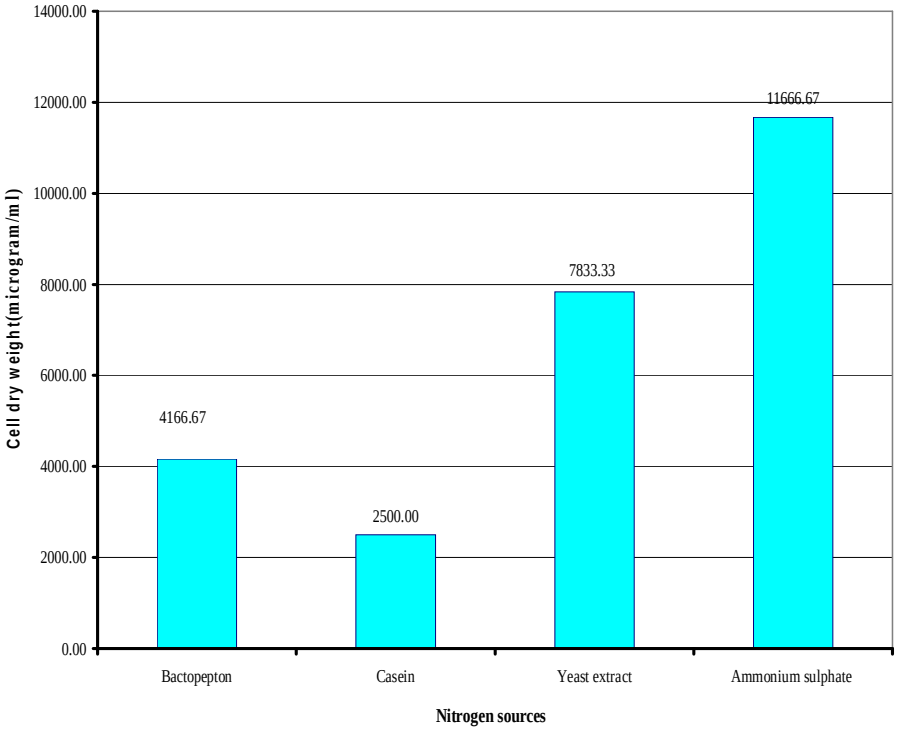

Figure1.Average of cell dry weight by isolated bacterium of *Azotobactervinelandii* with type of different nitrogen sources

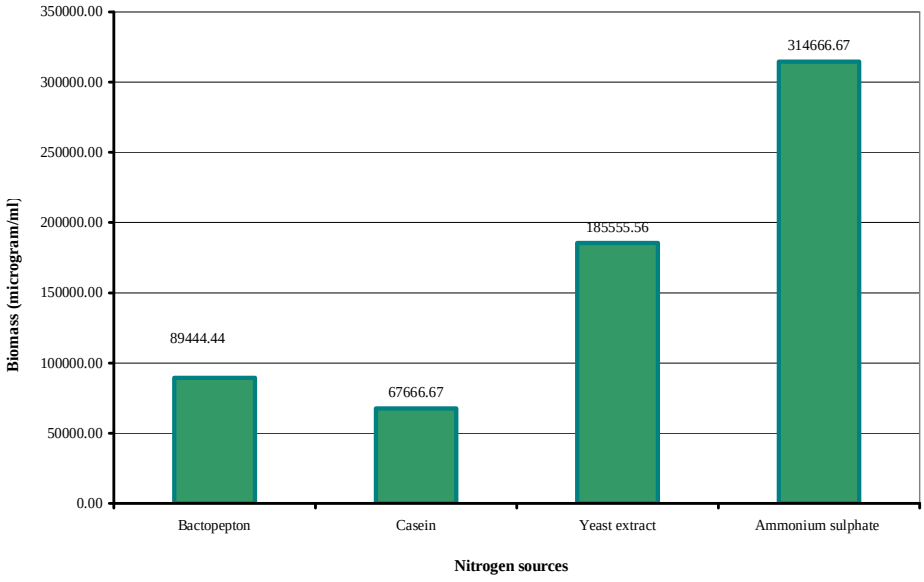

Figure2. Average of biomass by isolated bacterium of *Azotobacter vinelandii* with type of different nitrogen sources

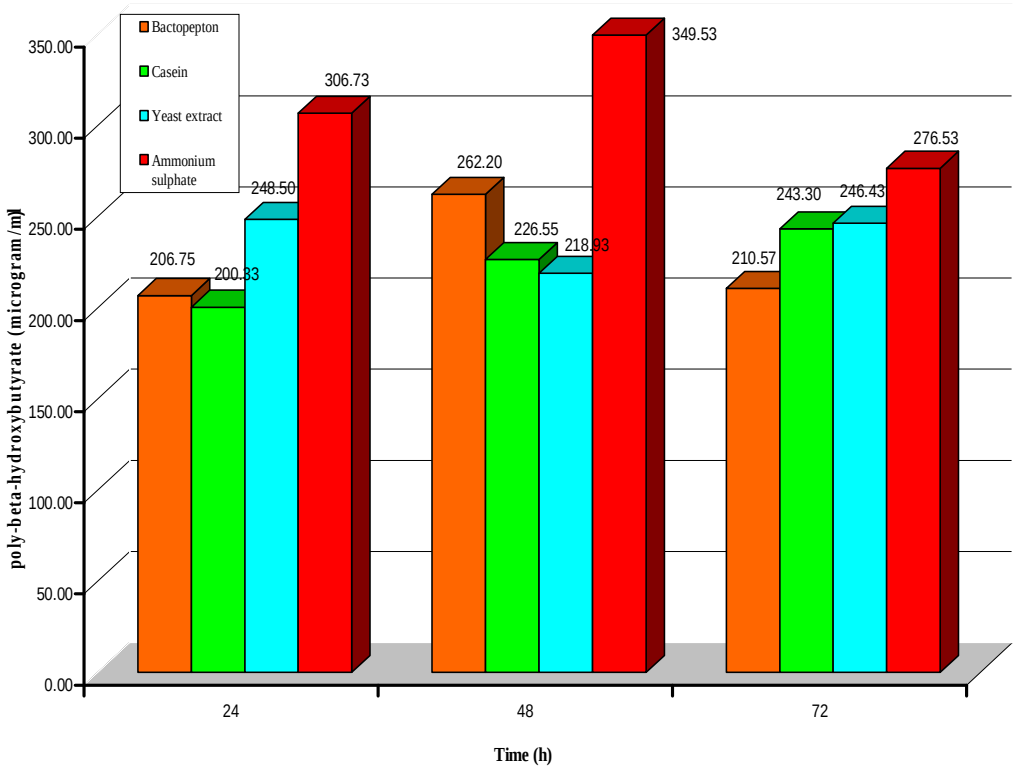

Figure3. Average of poly-ß-hydroxybutyrateby isolated *Azotobacter vinelandii* with type different of nitrogen sources in different times

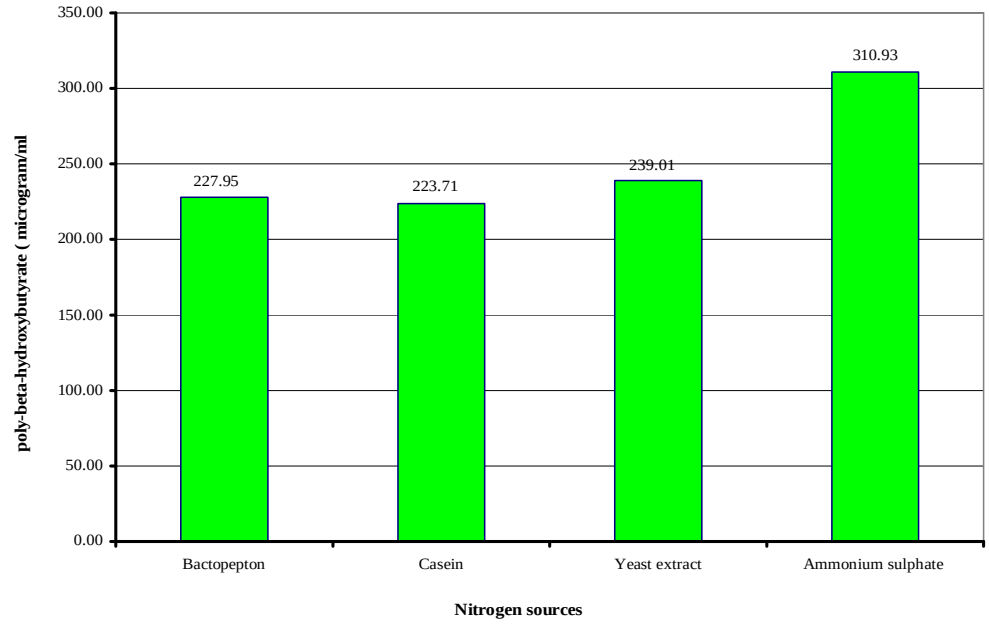

Figure 4. Production of poly-ß-hydroxybutyrate by *Azotobacter vinelandii* with type different of nitrogen sources in different times

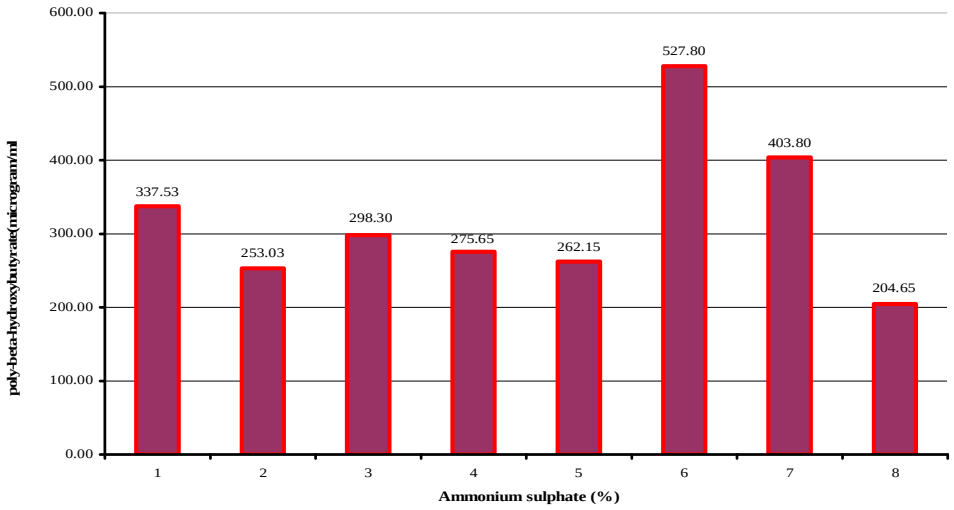

Figure 5. Production of poly-ß-hydroxybutyrate by *Azotobacter vinelandii* with different percentages of ammonium sulphate

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