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Biodegradable plastics from *Sinorhizobium meliloti* as plastics compatible with the environment and human health

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Abstract

Introduction: Polyhydroxyalkanoates (PHAs) are natural polyesters and biodegradable plastics that are stored as intracellular inclusion bodies by a great variety of bacteria. The aim of this study was to extract polyhydroxyalkanoate from native *Sinorhizobium meliloti* in Iran.

Materials and methods**:** *Sinorhizobium meliloti* isolates were collected from roots of alfalfa plants and were identified by Gram staining, biochemical experiments and amplification of 1500 bp fragment of *16Sr DNA* gene. PHA granules were detected by microscopic examination. PHA production was evaluated in nutrient deficient medium and its amount was determined by conversion of PHA into crotonic acid by sulphuric acid treatment. The effect of various temperatures, agitation rate and carbon source (sucrose, mannitol, and maltose) were evaluated on dry cell weight and polyhydroxybutyrate (PHB) production.

Results: The maximum amount of polymer production (43.10%) was seen in basal mineral medium at 29°C, pH~7 and 215 revolutions per minute (rpm). The results of this research showed that the S5 isolate was capable to produce maximum poly3- hydroxybutyrate. The produced polymer was analyzed for its purity by GC- mass (gas chromatography- mass spectroscopy) and confirmed to be PHB compared with the standard polymer.

Discussion and conclusion: Native strains of *Sinorhizobium* can be used in the production of biodegradable plastics and the results of present study showed that *S. meliloti* S5 was capable to produce maximum PHB at 29°C, agitation rate of 215 rpm, and pH~7.

Key words: Polyhydroxyalkanoate**,** *Sinorhizobium meliloti*, Biodegradable plastics, GC-mass

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Introduction

Synthetic plastics are one of the greatest mankind's inventions and have been developed into a major industry and indispensable commodity in humans' life (1). These materials, which are synthesized chemically by polymerization, contribute towards air pollution and waste management problems (2). Natural products are being developed as biodegradable plastics including starch blends, polylactic acid, and bacterial polyhydroxyalkanoates (PHA). PHA, most commonly represented by polyhydroxybutyrate (PHB), are natural polyesters that are stored as intracellular inclusions by a great variety of bacteria (3 & 4). Among these, PHA which belongs to the group of polyoxoesters has received intensive attention because it possesses biodegradable thermoplastic properties (5). PHA is synthesized by bacteria under unbalanced growth conditions. Some bacteria have been reported capable to produce PHA as much as 90% (w/w) of dry cells during depletion of essential nutrients such as nitrogen, phosphorus or magnesium (6).

Polyhydroxyalkanoates are converted to carbon dioxide and water in aerobic and to methane in anaerobic conditions by organisms living in soil, water and other habitats (7).

Rhizobia are nitrogen- fixing bacteria that have many benefits for plants. They can affect plant growth by producing and releasing secondary metabolites and facilitate the availability and uptake of certain nutrients from the root environment. Inoculation of Persian clover with *Rhizobium* strains could alleviate the effect of SO_2 pollution on antioxidant system by effects on root growth (8).

Regarding the irresolvablity of chemical plastics and their accumulation in the environment, there is a need for the research to develop new biodegradable alternatives to synthetic plastics. The aim of the present research was to extract polyhydroxyalkanoate from the native *Sinorhizobium meliloti* in Iran.

Materials and methods

Isolation and identification of bacteria: Bacteria were isolated from homogenized pink nodules of alfalfa plants roots. The suspensions were cultured in yeast extract mannitol agar (YEMA). Plates were incubated for 3- 4 days at 28ºC and suspected bacteria were re- streaked on YEMA agar containing 0.0025% congo red. *Sinorhizobium meliloti* PTCC 1684 taken from Persian Type Culture Collection in Iran was used as a standard strain. All isolates and standard strain were stored at 70°C, in yeast extract mannitol medium containing 25% (vol/ vol) glycerol. Bacteria were analyzed for colony morphology, Gram stain, motility and biochemical tests including catalase, oxidase and carbon source utilization (9).

In order to molecular identification, total genomic DNA of *Sinorhizobium* isolates were extracted using chloroform extraction and ethanol precipitation (10). Extracted DNAs were evaluated by using electrophoresis on 1% gel agarose and staining with green viewer along with 100

bp molecular weight ladder standard (Fermentas, Lithuania). The concentration and purity of DNA was estimated spectrophotometrically at 260- 280 nm. The extracted DNA was further used for the following PCR assay.

50- 80 ng of extracted DNA was used to perform PCR. Identification of 16S rDNA gene of *S. meliloti* was conducted with a pair of primers fD1 (5'- AGAGTTTGATCCTGGCTCAG- 3') and rD1 (5'- AAGGAGGTGATCCAGCC- 3') which amplified a 1500 bp fragment (11). PCR was performed in a thermal cycler (Kyratec, Singapore) with conditions as follow: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 90 sec and final extension 72°C for 5 min. A 100 bp DNA ladder (SM0323- Fermentase, Lithuania) was used as molecular weight marker. PCR products were separated on 1.5% agarose gel for 3 hour at 50 V followed by staining with green viewer and detection under Gel documentation system (Kimiagene- Iran). *Sinorhizobium meliloti* PTCC 1684 was used as a positive control.

Plant infection test: All isolated bacteria were tested for symbiotic relationship on Jensen- nitrogen free agar slants (12). The test was performed using sterile test tubes containing N- free medium added with 0.8% agar as plant growth medium. Alfalfa seeds variety Hamedani were surface sterilized before planting. Each tube was inoculated with 1 mL of broth culture containing 10^8 cells/mL at the time *root hair*s were being formed. Plants were watered with nitrogen free nutrient solution (13). A set of test tubes fertilized with seeds infected by standard strain and seeds without infection were included as controls.

Detection of PHA granules: PHA granules were detected by Phase- Contrast microscopy and sudan black staining. After drying smear and fixing by immersing in 2% acetic acid for 5 minutes, the slide was dried and covered with sudan black solution (0.3% in ethanol) for 15 minutes and was cleared by xylene. The smear was then stained with 0.5% aqueous safranin as a counter stain for 10 seconds. The slide was washed and dried and then seen under light microscope (14).

PHA production: Pure and fresh colonies of bacteria from YEMA were inoculated in 100 mL of sterile basal mineral medium containing (g/L) Na_2HPO_4, $2H_2O$ 4.4; KH_2PO_4 1.5; $(NH_4)_2SO_4$ 1.0; $MgSO_4$, $7H_2O$ 0.2; sucrose 10; yeast extract 0.5; pH 7.0 (2). The flasks were incubated at 29ºC in a shaker incubator with 215 rpm for 72 h. After 72 h, 1 mL of this culture was transferred into 100 mL sterile nutrient deficient medium including 20 g/L sucrose as carbon source and incubated at 29ºC and 215 rpm for 72 h. The cultures were harvested and subjected to further experiments.

***Sinorhizobium* growth curve:** 0.1 ml of each pure bacterial suspension (OD_{600} = 0.2) was inoculated to 10 mL of yeast extract mannitol broth (YEMB). Cultures were incubated at 37ºC for 72 hours. *Bacterial growth* was determined *using spectrophotometer* (Hitachi, Japan) taking optical density (OD) at 600 nm at every 12- hour intervals.

Estimation of biomass: Dry cell weight was estimated by centrifugation of 10 mL of 72 h old broth medium at 4000 rpm for 20 min followed by washing twice with distilled water and drying the precipitated cells at 60ºC. At last, the dried cells were accurately weighted (A&D- Japan) and recorded.

Crotonate assay: The amount of PHA was determined by conversion of PHA into crotonic acid by sulphuric acid treatment (15 & 16). For this, 10 mL of concentrated sulfuric acid was added to the dried biomass in screw cap tube and heated for 10 min at 100ºC in a water batch. Crotonic acid which was formed was measured at 235 nm. Poly [(R) - 3- hydroxy butyric acid], Sigma Aldrich USA was used as a standard. The percent of PHA of the sample was calculated using the fallowing formula (17): CDW is the amount of dried biomass in g/L.

PHB% = (Absorbance in 235 nm /CDW /10 mL) × 100

The effect of different factors for PHA production: To investigate the effects of different temperatures (25, 29, 33, 37 and 41ºC), agitation rate (180, 215 and 250 rpm) and carbon source (sucrose, mannitol, and maltose) on PHA production, the experiments were done same as above. Different nutrient limited media were prepared with different sugars that most of strains consume. All cultures were harvested and the growth was calculated by dry weight determination and the amount of PHAs produced was estimated by using crotonic acid assay.

Characterization of PHA by GC- Mass: Purity of polymer was also checked by GC-mass analysis (Variancp- 3800 gas chromatograph). PHA sample was subjected to methanolysis in sealed tubes at 100ºC for 15 min in a water batch. For methanolysis, 1 mL of chloroform, 0.85 mL of methanol- benzoic acid, and 0.15 mL of concentrated H_2SO_4 were used. Before analysis, distilled water was added to the sample and contents were shaken vigorously for 2 min and placed in a stationary position for 1 min. Methyl esters in the organic phase were analyzed (2 & 18). Calibration was performed using standard Poly [(R) - 3- Hydroxy Butyric Acid] (Sigma- Aldrich) with benzoic acid as internal standard.

Results

In this study, 8 isolates of *Rhizobium* bacteria were collected from nodules of *Medicago sativa* roots. All bacteria were Gram negative bacilli and coccobacilli, catalase and oxidase positive, motile and were able to utilize mannitol, maltose and sucrose as carbon source. After extracting chromosomal DNA, a fragment of 16S rDNA gene with a size of approximately 1500 bp was amplified using fD1 and rD1 primers. Fig. 1 shows electrophoresis of PCR products for *Sinorhizobium* bacteria along with 100 bp marker standard (SM0323) on 1.5% gel agarose. All bacteria created nodules in the infection test.

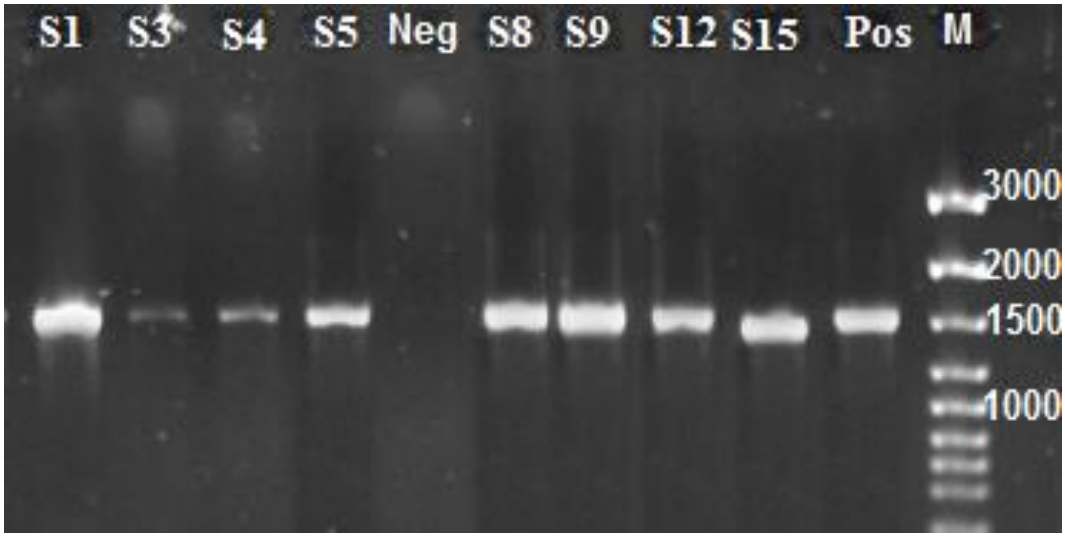

Fig. 1- Gel electrophoresis of *16S rDNA* gene PCR product of *Sinorhizobium meliloti* isolates (M: Marker, Neg: Negative control, Pos: Positive control, S1– S15: Samples)

PHB granules were seen by phase-contrast microscope in all bacteria. The slides stained with Sudan black were observed under light microscope and finally, from eight isolated bacteria, four isolates that showed significant PHA black granules were selected for further examinations.

Results of the mean optical density (growth curve) of rhizobial isolates have been shown in Fig. 2. The results showed that there were four different stages in growth curve of isolates including delay phase, exponential phase, stationary phase, and death phase. In this experiment, testing was stopped when the isolates were entered in death phase.

The effect of various temperatures was evaluated on cell dry weight and PHB production. As shown in Fig. 3 the optimal temperature for production of cell biomass and PHB was 29ºC. The highest cell biomass and PHB production was 42.70% at 29ºC.

Fig. 4 shows the effect of agitation rate on PHB production. As indicated in this figure the optimal shaking rate was 215 rpm and the highest percent of PHB production was 43.10% of cell dry weight. In higher shaking rate at 250 rpm, the percentage of PHB production was reduced.

The relation between PHB production and carbon source has been shown in Fig. 5. The highest rate of PHB production with 42.23% occurred in medium contained sucrose.

Among native isolated *Sinorhizobium* bacteria, S5 was found the best strain to yield the highest polymer of PHB.

PHB extracted from S5 was analyzed by GC (Fig. 6) for its purity and compared with standard PHB. There is a pick at region 71 (similar to the standard) in GC-mass chromatogram that indicates the presence of CO.

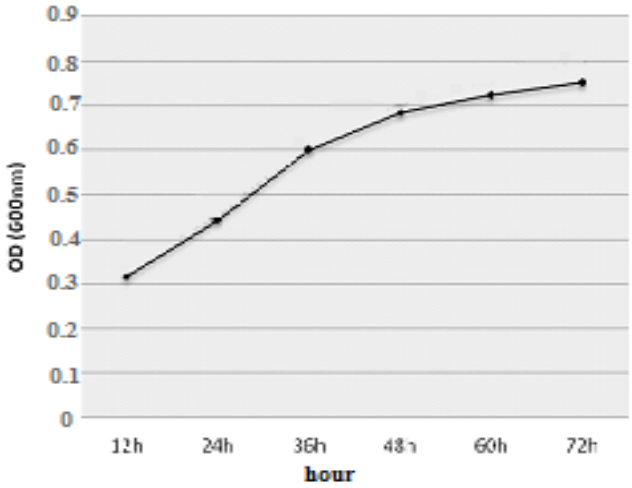

Fig. 2- Growth curve of native isolated *Sinorhizobium meliloti* after 72 hours of growth in YEMB

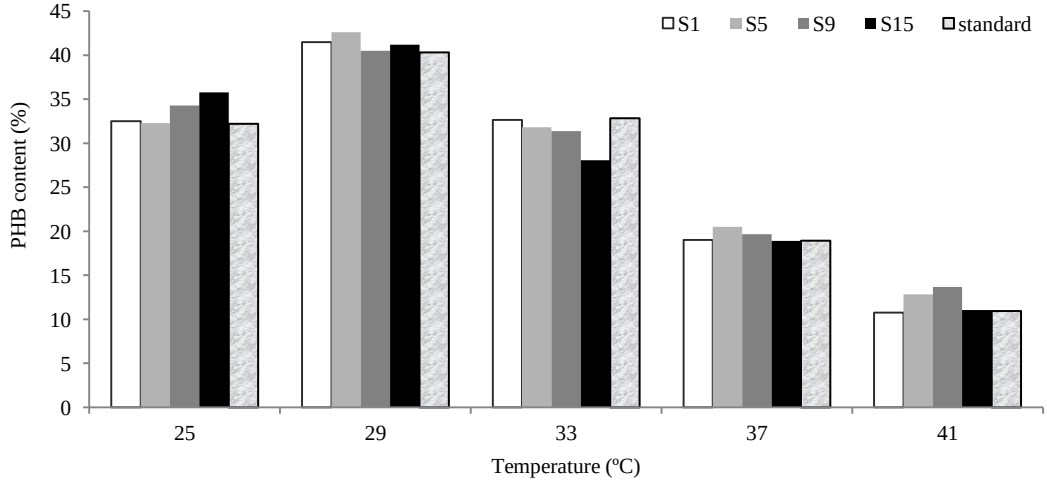

Fig. 3- The effect of different temperatures on PHB production for reference strain and four native *Sinorhizobium* isolates in Iran

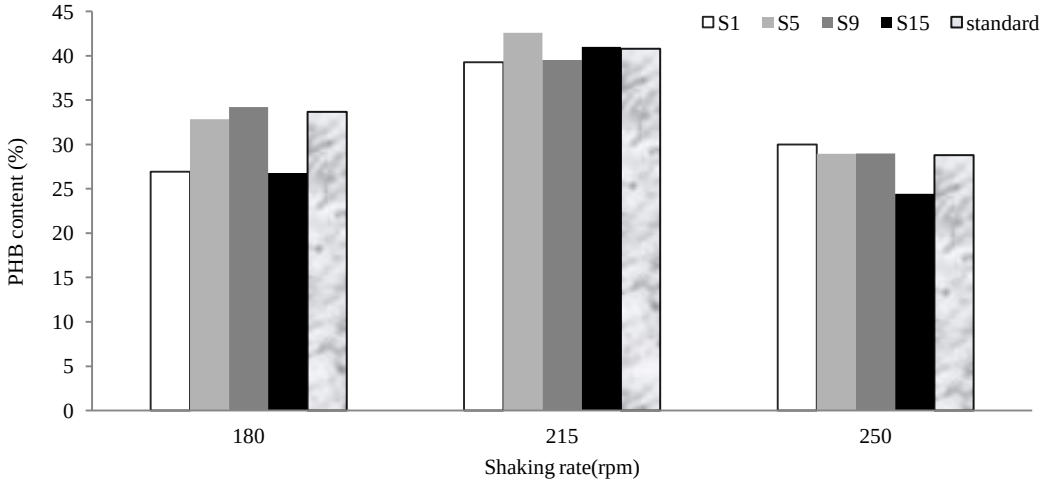

Fig. 4- Effect of various shaking rate on PHB production for reference strain and four native *Sinorhizobium* isolates in Iran

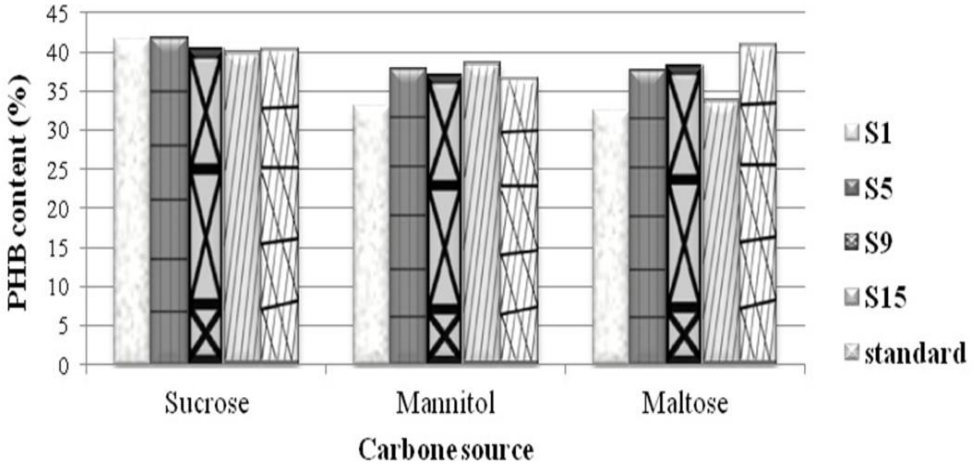

Fig. 5- The effect of different carbon source on PHB production for reference strain and four native *Sinorhizobium* isolates (S1, S5, S9, and S15) in Iran

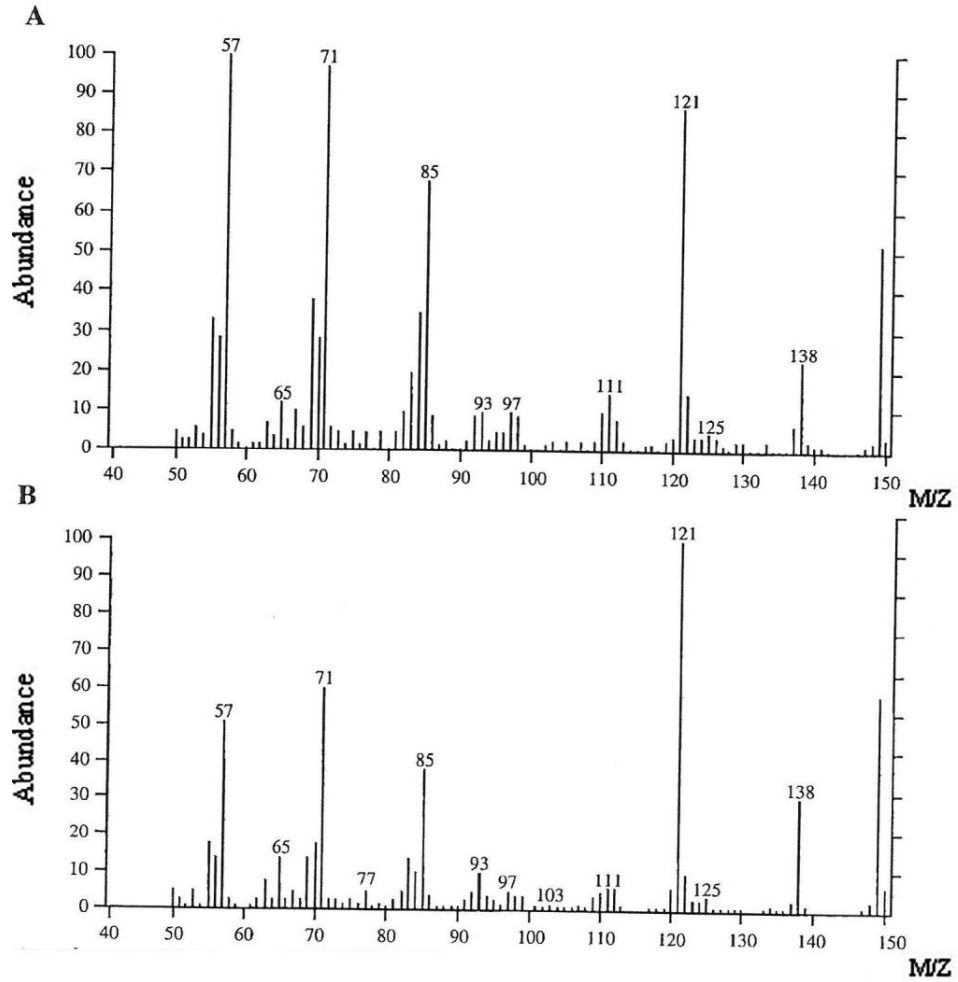

Fig. 6- GC mass spectrum of the standard PHB (A) and PHB extracted from *S. meliloti* S5 isolate (B).

Discussion and conclusion

Results showed that the PHB production increased with elevated agitation rate but by increasing agitation rate to 250 rpm, the PHB decreased. Oxygen is a studied important factor for PHB production in bacteria and has different effects on growth and polymer accumulation in different microorganisms. In this study, the highest rate of polymer production and also the highest amount of biomass were observed at 215 rpm. An explanation for these results is that reduced concentration of oxygen in active growth phase usually leads to excess production of NAD(P)H, that can be applied in fermentative mechanisms of energy to produce low molecular weight fatty acids. TCA cycle is also prevented by hypoxia because of citrate synthase enzyme inhibited by NAD(P)H activity. As a result of the above processes, the cofactor required for the synthesis of poly3-hydroxybutyrate synthesis is produced by most bacteria (19).

In other hand, bacterial growth at 180 rpm and 250 rpm is less than 215 rpm. So, we can conclude that despite the production of PHB is stimulated by lower concentration of oxygen, bacterial growth is decreased in low concentrations of oxygen. Thus, although oxygen limitation is an important factor to induce polymer formation, but oxygen reduction to a certain amount, influence on growth and will be followed by limitation of bacterial growth.

The results of this research showed that S5 isolate was capable to produce maximum poly3- hydroxybutyrate. This isolate exhibited maximum amount of polymer production (43.10%) in basal mineral medium at 29°C, pH 7 and 215 rpm. In this study, PHB production of the isolates was between 41 and 42.7% and this result was in agreement with those obtained by some other researchers. According to Tombolini and Nuti, the content of this polymer in rhizobia ranges from 30 to 55% of dry cell weight (20).

Kshama and Shamala extracted polyhydroxyalkanoate from *Sinorhizobium meliloti* cells using *Microbispora* culture filtrate. *S. meliloti* produced 50% PHA in the presence of sucrose as carbon substrate (2). Less production of PHB in the present study can be related to the extraction method, carbon source or tested strains. PHAs amount varies according to the carbon source and type of used microorganisms (21) and between 40 and 80 percent is variable (22). Possibly we can increase the amount of PBS production in these bacteria by changing the carbon source that requires further investigation.

In a study conducted by Mercan et al., the production of PHB was determined in strains of *Rhizobium* spp., *Rhizobium cicer* and *Bradirhizobium japonicum* and the content of PHB according to dry cell weight was determined to be 1.38 to 40% (23).

Aarthi and Romana reported that PHB production of strains of *Bacillus cereus* and *B. mycoides* were found to vary from 12.18 to 57.2% of dry cell weight. The highest PHB yield was observed in *B. mycoides* DFC1 accumulating as high as 1.83 g/L. Studying on polymer production using several complex carbon source showed that

B. mycoides DFC1 resulted in a high PHB yield in wheat starch containing medium at the end of 48 h of growth. The extracted polymer showed the intense absorbance characteristic for ester carbonyl (C=O) stretching group at 1720 cm^{-1} (24).

This is the first report of the extraction of biodegradable plastics from *Sinorhizobium meliloti* native bacteria in Iran. Biodegradable plastics from bacteria are valuable and can be used to produce medical apparatus compatible with human body. We concluded that *S. meliloti* S5 was capable to produce maximum poly3-hydroxybutyrate and the optimum condition for more production was temperature incubation at 29°C, agitation rate of 215 rpm, and pH~7.

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پلاستیک‌های قابل تجزیه از باکتری‌های *سینوریزوبیوم ملیلوتی* به عنوان پلاستیک‌های سازگار با محیط و سلامت انسان

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چکیده

مقدمه: پلی‌هیدروکسی آلکانوآت‌ها، پلی استرهای طبیعی و پلاستیک‌های قابل تجزیه هستند که در دانه‌های ذخیره‌ای انواع متنوعی از باکتری‌ها ذخیره می‌شوند. هدف از این پژوهش استخراج پلی هیدروکسی آلکانوآت (PHA) از باکتری‌های بومی *سینوریزوبیوم ملیلوتی* در ایران بود.

مواد و روش‌ها: جدایه‌های *سینوریزوبیوم ملیلوتی* از ریشه‌های گیاهان یونجه جمع آوری و با رنگ آمیزی گرم، آزمایشات بیوشیمیایی و تکثیر قطعه ۱۵۰۰ جفت بازی از ژن *16Sr DNA* شناسایی شدند. گرانول‌های PHA با آزمایش میکروسکوپی، ردیابی شدند. تولید PHA در محیط با کمبود مواد غذایی ارزیابی شد و مقدار آن با تبدیل PHA به کروتونیک اسید به‌وسیله تیمار سولفوریک‌اسید تعیین شد. اثر درجه حرارت، میزان هم زدن و منبع کربن (ساکاروز، مانیتول و مالتوز) بر وزن خشک سلول و تولید PHA بررسی شد.

نتایج: حداکثر مقدار تولید پلیمر (۴۳/۱۰ درصد) در محیط پایه معدنی در ۲۹ درجه سانتی‌گراد، اسیدیته حدود ۷ و دور ۲۱۵ دور در دقیقه (rpm) مشاهده شد. نتایج این پژوهش نشان داد که جدایه S5 قادر به تولید بیش‌ترین مقدار پلی۳ هیدروکسی بوتیرات بود. پلیمر تولید شده از نظر خلوص با روش گازکروماتوگرافی (GC- Mass) تحلیل شد و از نظر پلی هیدروکسی بوتیرات (PHB) در مقایسه با پلیمر استاندارد تایید شد.

بحث و نتیجه‌گیری: می‌توان از سویه‌های بومی *سینوریزوبیوم* در تولید پلاستیک‌های قابل تجزیه استفاده کرد. نتایج این پژوهش نشان داد که جدایه ۵ *سینوریزوبیوم ملیلوتی* در ۲۹ درجه سانتی‌گراد، دور همزن ۲۱۵ rpm و اسیدیته حدود ۷ قادر به تولید بیش‌ترین مقدار تولید PHB بود.

واژه‌های کلیدی: پلی هیدروکسی آلکانوآت، *سینوریزوبیوم ملیلوتی*، پلاستیک‌های قابل تجزیه، گاز کروماتوگرافی جرمی

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