

## ***Bacillus thuringiensis* strain NG, a Novel Isolated Strain for production of Various Polyhydroxyalkanoates**

**Nazila Gholamveisi**

M.Sc Student of Microbiology, Department of Biology, College of Basic Sciences, Islamic Azad University of Sanandaj  
n.gholamveisi@gmail.com

**Sirvan Mohammadi Azar**

Assistant Professor of Chemistry, Department of Chemistry, College of Basic Sciences, Islamic Azad University of Sanandaj  
mohammadiazar@iausdj.ac.ir

**Roya Moravej\***

Instructor of Microbiology, Department of Biology, Faculty of Science, Islamic Azad University of Sanandaj roya.sanandaj@gmail.com

### **Abstract**

**Introduction:** Microbial biopolymers such as polyhydroxyalkanoates (PHA) are proper alternatives for petroleum-derived plastics. These biopolymers have many advantages over conventional plastics such as biodegradability, environmental friendly and infinite as a renewable resource. Therefore, our study was aimed to isolate a bacterial strain capable of producing polyhydroxybutyrate (PHB; a highly applicable type of PHA).

**Materials and methods:** To this aim, a total of 6 PHA-producing bacteria were isolated from waste water exit site of a brewery factory. The 6 isolates were studied by Sudan black-staining technique and the most stained isolate was selected for further studies. Next, the selected isolate was identified based on morphological, biochemical and phylogenetic analyses. Finally, the ability of strain in producing PHB as well as other PHAs was analyzed via GC-MS technique.

**Results:** Strain NG had the highest yield of PHB, according to Sudan black-staining technique and it was selected for further studies. The strain NG was identified as a new strain of *Bacillus thuringiensis*. According to GC-MS results, this strain was able to produce PHB as well as 4 other PHAs including hexadecanoic acid methyl ester, octadecanoic acid methyl ester, tetradecanoic acid methyl ester, 8-octadecenoic acid methyl ester.

**Discussion and conclusion:** It was the first report on producing various PHAs at the same time by a strain of *Bacillus thuringiensis*.

**Key words:** *Bacillus thuringiensis* NG, Polyhydroxyalkanoates, polyhydroxybutyrate, GC-MS

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\*Corresponding Author

## Introduction

Nowadays, Plastics are almost used in every industry and it is estimated that over 240 million tons of plastics are produced globally every year, 44% of which enters the environment in the same year, e.g. marine environment, forest area and farmlands (1). Most of these plastic materials are made from petroleum derivatives such as polyethylene and are resistant to degradation and remain in those environments even for centuries, leading to wide pollutions which are dangerous for living organisms (2). The main reason for durability of plastics in the environment is high molecular weight of its polymers, strong chemical bonds and lack of biological enzymes which can catabolize them (3, 4).

Elimination of plastics from the environment via chemical and physical approaches such as burning them is one of the solutions toward the issue. However, these approaches are expensive, time consuming or dangerous, occasionally. Moreover, more plastics are produced again and all of those efforts and costs will diminish (5). On the other hand, petroleum resources are finite and suitable alternatives are critically needed. Microbial biopolymers seem suitable alternatives for conventional plastics. These biopolymers are polyesters which are found in nature as intracellular compartments of some types of micro-organisms and act as a source of energy for them (6).

Among the microbial biopolymers, a group called polyhydroxyalkanoates (PHA) has got much attention in recent years because of their numerous advantages, including 100% biodegradability, flexibility, water resistance, low cost of production and simplicity of synthesis process (7). PHAs are naturally occurring polyesters produced by bacteria via fermentation of sugars or lipids and include more than 100 types of various compounds

with extremely different properties (8). PHA synthases are the main enzymes responsible for production of PHAs which use coenzyme A-thioester of (r)-hydroxy fatty acids as their substrate. These hydroxy fatty acids will turn into monomers of PHAs. In fact, extremely different properties of PHAs are due to the length of the monomers, number of the monomers and being homo- or copolymers (9, 10).

Polyhydroxyalkanoates can be produced by a variety of bacteria including *Bacillus*, *Azotobacter*, *Pseudomonas*, *Alcaligenes*, and *Klebsiella*, under a particular condition (low nitrogen, phosphorus, sulfate and excess levels of carbon resources, Simultaneously) (11). Some of these bacteria are used for industrial production of PHA. However, PHA type and content yielded by these bacteria are not the same. In fact, the efficiency of the process depends on many factors, including microorganism type, the substrates used, temperature, pH, and other culture conditions (12, 13). Therefore, it is of great importance to find high-yield species and strains of bacteria and set up their optimal condition to use their substrate efficiently to produce PHAs. On the other hand, one of the main problems in industrial production of PHAs is related to its high costs which makes it much more expensive compared to petroleum-derived polymers. A great portion of the costs is related to supply suitable substrates (14, 15). Thus, using inexpensive and high-yield substrates can help expanding the use of such green materials and reduction of petroleum materials usage. In this regard, a potential resource of substrates of PHAs is waste water of beverage industries (16).

According to above literature, our study was aimed to isolate high-yield poly-3-hydroxybutyrate (a highly applicable type of PHA) producing bacteria from waste water of brewery industries.

## Materials and methods

**Collecting samples:** Samples were collected from water, soil, and sludge of waste water exit site of a brewery factory (Istak Factory, Sanandaj City, Kurdistan Province, Iran) regarding sterile condition. The samples were transferred to laboratory on ice immediately and stored at  $-20^{\circ}\text{C}$  until used.

**Enrichment and purification of bacteria:** 2 gr of soil or sludge samples and 2 ml of water sample were inoculated in final volume of 50 ml of Luria Bertani (LB) broth (Merck, Germany) in 100 ml Erlenmeyer flasks, separately. Next, the flasks were incubated at  $20^{\circ}\text{C}$  and 150 rpm for 24 h. After that, 0.1 ml of the flasks contents were transferred to LB agar mediums with different serial dilutions ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ) and bacterial isolation was performed by repetitive culturing on LB agar plates, to obtain pure single colonies (17).

**Isolation of PHB producing bacteria:** Lipophilic dyeing with Sudan Black B (Merck, Germany) which has a high affinity for PHB granules was performed to screen the PHB-producing bacteria. For this purpose, bacterial smears were prepared on microscopic glass slides, dried and fixed. Then, the slides were sunk in Sudan Black B 3% solution (w/v in 70% ethanol) for 10 min. Next, the slides were washed under running water and dried. The slides were counterstained with safranin (5% w/v in distilled water, Merck, Germany) for 10 seconds, washed again with running water, and dried. Finally, the slides were studied under 1000x magnification by optical microscope (Olympus CX-31, Germany) (18).

**Cryopreservation:** In order to preserve isolated PHB-producing bacteria to repeat experiments and further studies, their reserves were prepared. For this purpose, 0.3 ml of glycerol was added to 0.7 ml of 24 h culture of isolates (in LB broth medium) in cryo-vials. The cryo-vials

(simport, Canada) were then transferred to cryoboxes (sigma, U.S) and placed in  $-80^{\circ}\text{C}$  to reduce temperature, gradually. These cryo-vials were then transferred to nitrogen tank until used.

**Identification of the isolates:** The isolates were analyzed in terms of morphology of colonies, gram staining, and biochemical tests (macconkey, catalase, oxidase, citrate, starch hydrolysis,  $\text{H}_2\text{S}$ , indole, motility, VP, MR, TSI, nitrate tests). The results were analyzed using standard identification procedures in bacteriology and Bergey's manual of systematic bacteriology (9th edition) (19). At the next step of identification of the isolates, phylogenetic analysis based on 16S rDNA was done. To this aim, firstly, genomic DNA was extracted using High Pure PCR Template Preparation Kit (Roche, Germany, Product # 11796828001). Then, Taq PCR Master Mix Kit (Qiagen, Netherland, Cat No./ID: 201443) was used to amplify 16S rDNA fragment, according to manufacturer protocol. Two general primers including 16S F27: 5'-AGAGTTTGATCMTGGCTCAG-3' and R1492: 5'-TACGGYTACCTTGTTACGACTT-3' were used as forward and reverse primers, respectively (20). PCR reaction was run as follow: 1) initial denaturation at  $95^{\circ}\text{C}$  for 5 minutes 2) denaturation at  $95^{\circ}\text{C}$  for 60 seconds 3) annealing at  $56^{\circ}\text{C}$  for 60 seconds 4) extension at  $72^{\circ}\text{C}$  for 2 minutes. Steps 2- 4 were repeated for 35 cycles. A final extension was run at  $72^{\circ}\text{C}$  for 2 minutes. PCR products were run on 1% agarose gel electrophoresis system and stained with ethidium bromide. The stained gel was then visualized under UV using a gel documentation system (Bio-Rad Gel Doc 1000). Next, PCR products were sent to CinnaGen Company (Tehran, Iran) for DNA sequencing. Finally, the DNA sequences were edited using BioEdit software, compared with other 16S rDNA

sequences available on NCBI database using Blast software and phylogenetic tree were drawn by Mega 7 software.

**Gas chromatography-Mass spectrophotometry (GC-MS) analysis:** GC-MS technique was used for determination of the PHB produced by isolates. For this purpose, firstly, a suspension of 20 mg of dried cells (obtained from 24 h culture on N2 deficient nutrient broth medium), 2 ml chloroform, 750  $\mu$ l methanol (0.4 mg/ml benzoic acid as internal standard), and 150  $\mu$ l sulfuric acid was prepared in tubes with Teflon-lined cap for each isolate. These tubes were placed in bain-marie at 100°C for 2.5 hours to ensure esterification reactions. Then, the tubes were cooled down to room temperature, 1 ml Deionized water was added to them and vortexed for 1 min. After these procedures, 3 distinct phases were formed (upper, middle and bottom phase contained sulfuric acid, cellular debris and Hydroxyalkanoate Methyl Esters, respectively). Upper and middle phases were disposed and bottom phases were transferred to cryo-tube and placed in -80°C until use. To prepare an external standard, 20 mg of PHB standard (Sigma, US) was used instead of dried cell and went under the procedure explained above. The bottom phase (organic phase) was analyzed by a gas chromatography mass system (Agilent 7890B GC System/ 5977A MSD) with capillary column HP-5. The initial oven temperature was set on 60°C which was increasing 10°C/min until 150°C. The temperature was then increasing 35°C/min until reached 220°C. The carrier gas was helium with 34 psi pressure and flow rate of 1 ml/min (17). We also determined the isolate potential to produce some other PHA compounds with the same manner.

## Results

**Screening of PHA-producing bacteria on LB culture:** 17 bacterial colonies were isolated from enrichment on LB cultures, among which 6 were considered as PHA-producing. Sudan black and GC-mass results indicated the isolate called NG (NG is an abbreviation of first author's name and is published on NCBI website) was the most efficient isolate to produce PHB. Therefore, NG was selected for further studies.

**Morphological and biochemical results:** Isolate NG was investigated based on its morphological, microscopic and biochemical features according to Bergey's Manual of Determinative Bacteriology. NG had creamy white, smooth, round colonies on nutrient agar which indicated that they are aerobic bacteria (Fig 1.A and Fig 1.B). Gram-staining test results also showed deep purple (gram positive) bacilli form bacteria (Fig 1.C). The results of biochemical tests are summarized in Table 1, which in sum indicated species belonging to *Bacillus* genus.

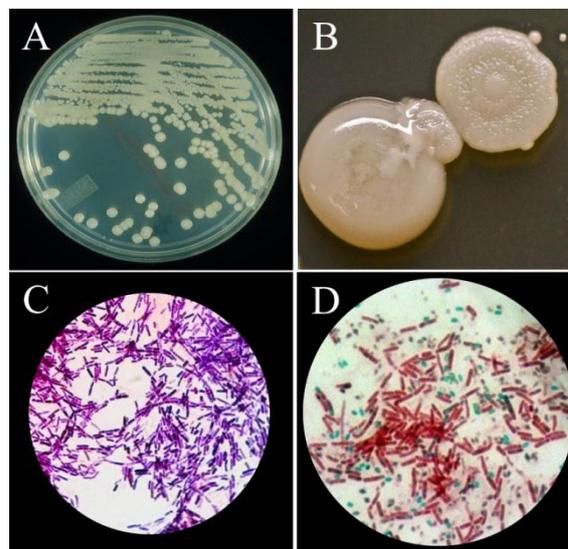


Fig 1- Results of morphological analyses. (A) plate culture of the isolate NG on nutrient agar. (B) colony appearance of the isolate NG (C) microscopic view of Gram stained NG (x1000) (D) microscopic view of Sudan black stained NG (x1600)

**Phylogenetic analysis:** Phylogenetic analysis showed that The isolate NG (GeneBank Accession No. KY657578) was a member of the genus *Bacillus*, and its closest relative was *B. thuringiensis* (GeneBank Accession No. KY368122) with 100% similarity (Fig. 2).

**GC-MS results:** GC-MS results revealed that the isolate NG was able to produce

PHB. Interestingly, it was observed that NG was also able to produce some other PHAs in remarkable (Fig 2). Amounts of the PHB and other PHAs produced by NG were reported as percentages of internal control (the area under PHB peak/the area under Benzoic acid peak) which are summarized in table 2 (Table 2).

Table 1- Biochemical tests for NG isolate

Biochemical Test	Result	Biochemical Test	Result
Catalase	-	Glucose	+
Oxidase	+	Sucrose	-
Citrate	-	Lactose	-
H <sub>2</sub> S	-	MR	+
Motility	-	VP	-
Indole	-	MacConkey Agar	-
Starch Hydrolysis	+	Nitrate	+

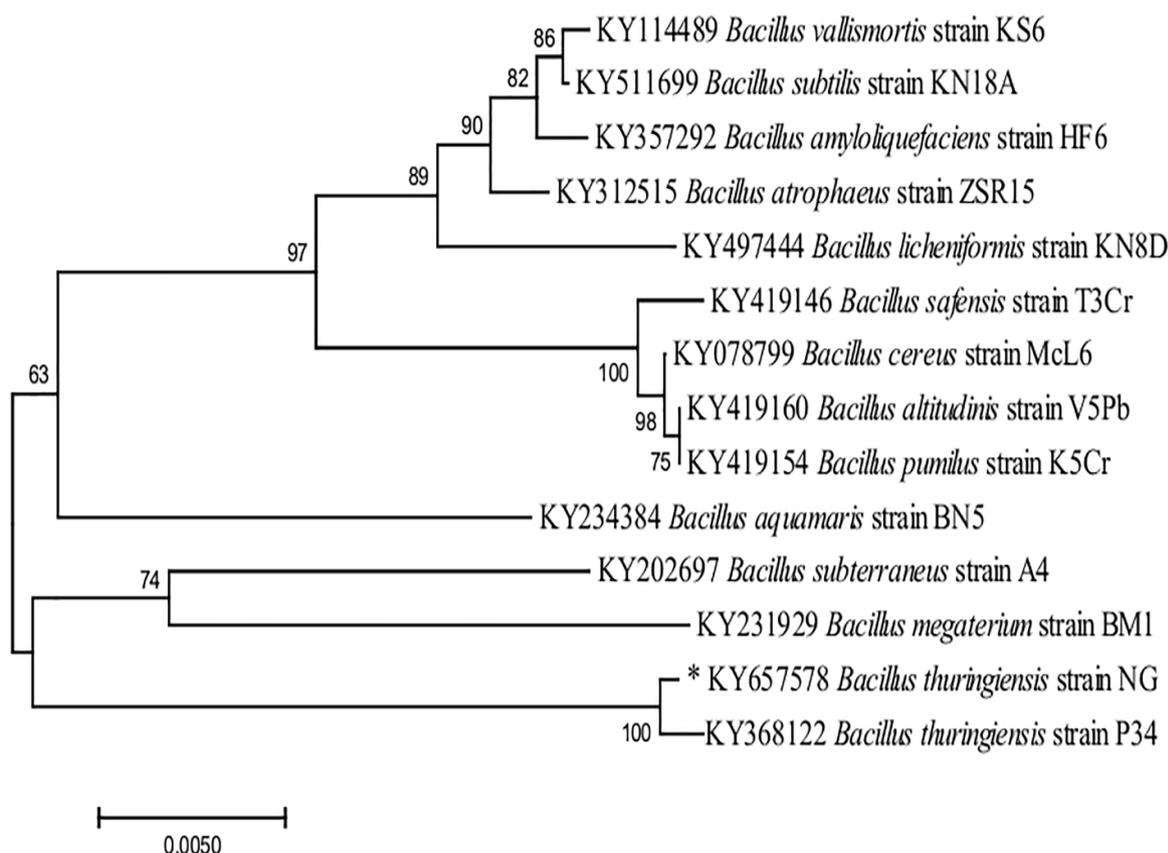


Fig 2. Phylogenetic tree obtained through neighbor-joining analysis of the 16S rRNA gene sequences using Mega 7 software. The position of the strain NG is shown within the genus *Bacillus*. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are given at the branch points. Scale bar represents 0.005 substitutions per nucleotide position. GeneBank accession numbers are shown in front of each strain. Strain NG is shown with \* sign.

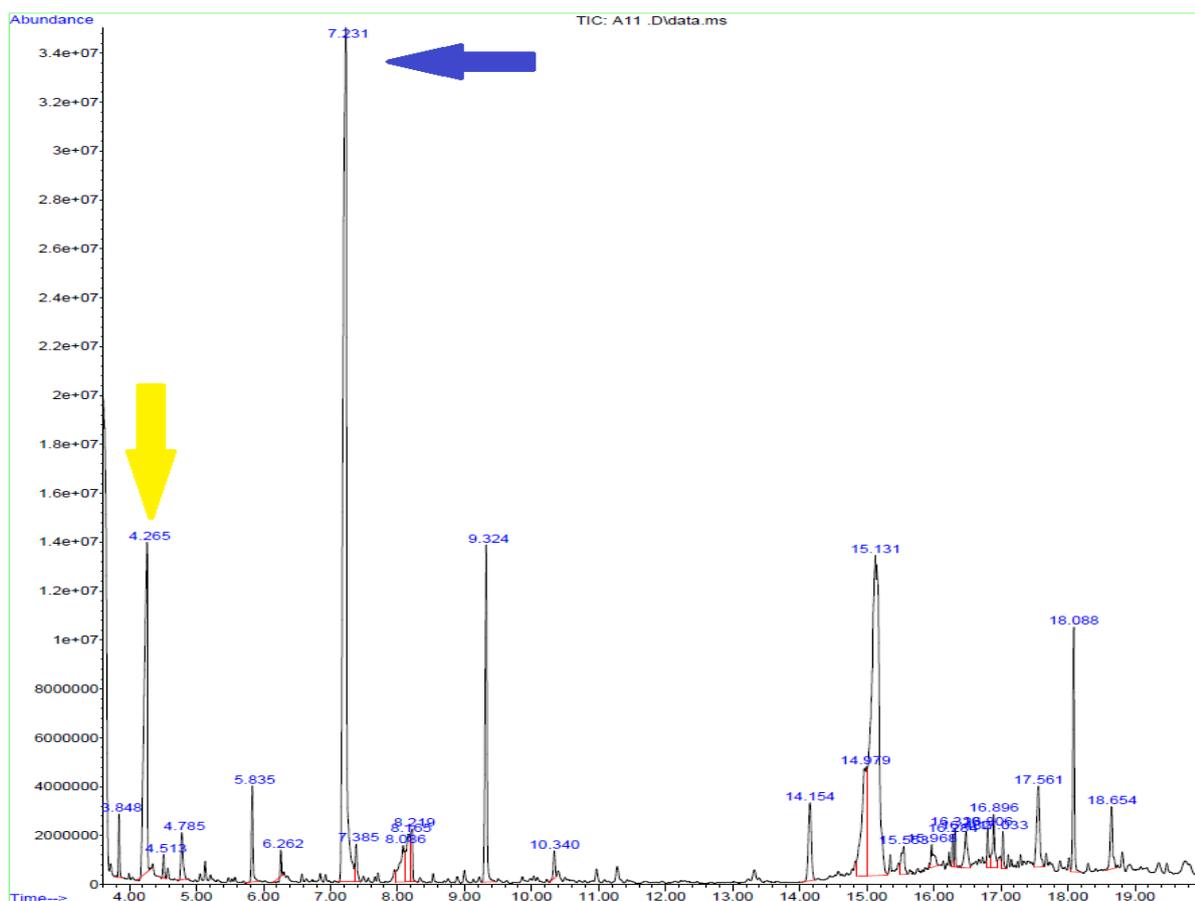


Fig 3. Diagram of GC mass analysis. The yellow arrow indicates PHB peak, while the blue arrow indicates Benzoic acid (internal control) peak. Other peaks are related to other NG PHAs detected by GC mass.

Table 2. amount of PHB and other PHAs produced by NG strain.

Methyl-esters	Amount produced by NG (% of Control)
Butanedioic acid, dimethyl ester (PHB)	33
Hexadecanoic acid, methyl ester	219
Octadecanoic acid, methyl ester	39
Tetradecanoic acid, methyl ester	26
8-Octadecenoic acid, methyl ester	24

## Discussion and conclusion

Biochemical and molecular studies were quite consistent with each other and identified the isolate NG as a new strain of *Bacillus thuringiensis*. This strain was submitted and registered on NCBI as a new strain (Gene bank accession number KY657578). *B. thuringiensis* is a well-known species in biotechnological and

agricultural studies. *B. thuringiensis* has a high ability to kill crop pests. In fact, its Bt genes are widely used to develop genetically modified crops. But genes are responsible to produce  $\delta$ -endotoxins which has insecticidal actions (21).

Strain NG was able to produce PHB in a remarkable level. In this regard, previous studies also showed *B. thuringiensis* that

can produce PHB (22, 23). The interesting point in our study was that we showed Strain NG could also produce relatively high amounts of other PHAs including Hexadecanoic acid methyl ester, octadecanoic acid methyl ester, tetradecanoic acid methyl ester, 8-octadecenoic acid methyl ester. Each of these compounds are of great industrial importance. This feature makes *B. thuringiensis* a multi-purpose bacterium which is quite useful for biotechnological and industrial applications. It is noteworthy that although current study was aimed to isolate a bacterial strain to produce PHB, Hexadecanoic acid methyl ester was the most produced PHA by strain NG.

As we mentioned earlier in introduction of this article, a great portion of costs in production of PHAs using bacterial approaches is related to supply suitable substrates. An effective bacterial strain which can use cheap and abundant carbon sources to produce PHAs is highly desirable. However, we didn't examine the ability of strain NG to grow on various carbon sources and the amount of PHAs produced on each, but previous studies showed *B. thuringiensis* is able to produce PHAs using agricultural wastes (24, 25). Additionally, *B. thuringiensis* can be isolated from various sources such as leaf surfaces, flour mills and grain-storage facilities (26). This, in turn, probably indicates that the bacterium is able to use a wide range of carbon sources. Despite these abilities and studies, efficiency of various carbon sources in production of PHAs using *B. thuringiensis* under different culture conditions has not been studied, universally. Therefore, for further studies, these factors are strongly recommended to be checked out.

This study clearly revealed that the isolate NG as a new strain of *B. thuringiensis* could produce PHB as well as some other valuable PHAs in significant

amounts. Also, it was the first report on producing various PHAs at the same time by a strain of *Bacillus thuringiensis*. Therefore, it can be considered as a potential strain for biotechnological purposes in the field of PHAs production. However, it is recommended to optimize the culture condition for maximum production of PHB in future studies.

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## سویه NG باسیلوس تورنجینسیس، یک سویه جدا شده جدید برای تولید انواعی از پلی‌هیدروکسی آلکانوات‌ها

نازیلا غلام ویسی: دانشجوی میکروبیولوژی، گروه زیست‌شناسی، دانشگاه آزاد اسلامی واحد سنندج، سنندج، ایران، n.gholamveisi@gmail.com  
 سیروان محمدی آذر: استادیار شیمی، گروه شیمی، دانشگاه آزاد اسلامی واحد سنندج، سنندج، ایران، mohammadiazar@iausdj.ac.ir  
 رویا مروج\*: مربی میکروبیولوژی، گروه زیست‌شناسی، دانشگاه آزاد اسلامی واحد سنندج، سنندج، ایران، roya.sanandaj@gmail.com

### چکیده

**مقدمه:** بیوپلیمرهای میکروبی مانند پلی‌هیدروکسی آلکانوات‌ها جایگزین‌های مناسب پلاستیک‌های مشتق از مواد نفتی هستند. این بیوپلیمرها مزایای بسیاری نسبت به پلاستیک‌های معمولی دارند، از جمله زیست‌تجزیه‌پذیری، سازگاری با محیط‌زیست و تجدیدپذیر بودن منابع آنها. از این رو، مطالعه حاضر با هدف جداسازی سویه باکتریایی دارای قابلیت تولید پلی‌هیدروکسی بوتیرات، یکی از پلی‌هیدروکسی آلکانوات‌های پرکاربرد، انجام شد.

**مواد و روش‌ها:** ۶ سویه باکتریایی تولیدکننده پلی‌هیدروکسی آلکانوات از محل خروجی پساب کارخانه تولید نوشیدنی جداسازی و با روش سودان بلاک مطالعه شدند. رنگ‌پذیرترین سویه برای مطالعه‌های بعدی انتخاب، بر اساس بررسی‌های مورفولوژیکی، بیوشیمیایی و فیلوژنتیکی شناسایی و توانایی آن در تولید پلی‌هیدروکسی بوتیرات و دیگر پلی‌هیدروکسی آلکانوات‌ها با روش GC-MS ارزیابی شد.

**نتایج:** بر اساس نتایج روش سودان بلاک، سویه NG بیشترین میزان PHB را تولید کرد و برای مطالعه‌های بعدی انتخاب شد. سویه NG، سویه جدیدی از باسیلوس تورنجینسیس شناسایی شد که بر اساس نتایج GC-MS، قادر به تولید پلی‌هیدروکسی بوتیرات و ۴ نوع پلی‌هیدروکسی آلکانوات دیگر شامل هگزادکانوئیک‌اسیدمتیل‌استر، اکتادکانوئیک‌اسیدمتیل‌استر، تترادکانوئیک‌اسیدمتیل‌استر و ۸-اکتادکانوئیک‌اسیدمتیل‌استر بود.

**بحث و نتیجه‌گیری:** مطالعه حاضر، نخستین گزارش از تولید هم‌زمان چند نوع پلی‌هیدروکسی آلکانوات توسط سویه‌ای از باسیلوس تورنجینسیس است.

**واژه‌های کلیدی:** باسیلوس تورنجینسیس NG، پلی‌هیدروکسی آلکانوات‌ها، پلی‌هیدروکسی بوتیرات، GC-MS

\* نویسنده مسؤول مکاتبات