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Full Length Article

Antibiotics Sensitivity and Heavy Metals Resistance in PHB-Producing Bacilli Isolated from Eastern Province, Saudi Arabia

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Abstract

There has been considerable interest in development and industrial production of polyhydroxybutyrate (PHB) biopolymers from bacteria due to its potential applications in agriculture, health and medicine. However, it is recommended to evaluate PHB-producing bacteria regarding sensitivity to antibiotics, resistance to heavy metals and possible toxicity before large scale production of the biopolymer. In this study, 120 bacterial strains isolated from different soil and sewage water samples in Eastern Province, Saudi Arabia, and screened for PHB production. Sixteen strains showed positive results with Sudan Black B and Nile Red A stains. 16S DNA gene analysis revealed high homologies to members of *Bacillus cereus* group, *B. megaterium*, *B. flexus*, *B. endophyticus*, and *B. aryabhattai*. For antibiotic profiling; *B. cereus* DS3-16 and *B. thuringiensis* DST1-33 showed highest resistance, especially to ampicillin, erythromycin and amoxicillin, while the least were *B. megaterium* DG8, DP7 and *B. pseudomycoides* DWST2-1. *Bacillus* sp. DSG5 and DPS6 followed by DSWT2-1, DS3-17 and DS3-12 showed highest resistance to Cr^{+6} (10 mg/mL), as well as Hg (62.5 µg/mL) and Ag (5 mg/mL), respectively while most strains were highly susceptible to other metals. Four isolates including *B. cereus* strain DS3-16 showed β-hemolysis activity, while *Bacillus axarquiensis* DSG5 showed α-hemolytic activity, indicating their possible toxicity. © 2016 Friends Science Publishers

Keywords: PHB biopolymer; Antibiotic sensitivity; Heavy metal resistance; Profiling; *Bacillus* sp.

Introduction

There is an increasing demand for production of many bio-based products including biopolymers to be used in agriculture, health, and medicine. Decades have been devoted on extensive research to develop biodegradable polymers as a promising substitute for petrochemical-based polymers due to their eco-friendly nature (Ojumu *et al.*, 2004). Among most commonly known bio-products is the bioplastic. When carbon source nutrients are available in excess relative to other nutrients in the growth environment, certain strains of bacteria produce and accumulate different types of polyester recognized as bioplastics, which have unique characteristics like biodegradability and biocompatibility (Madison and Huisman, 1999; Kim and Lenz, 2001; Steinbuchel, 2001; Ojumu *et al.,* 2004; Reddy *et al.,* 2009; Nayak *et al.,* 2013). Among widely known bioplastics, polyhydroxybutyrate (PHB) is the most prevailing polyester owing to its great resemblance to synthetic petroleum–derived synthetic plastic polymers (Mokhtari-Hosseini *et al.,* 2009; Berekaa and Al-Thawadi, 2012). Bioplastics have diversity of applications in medicine, tissue engineering, veterinary uses, agriculture, as well as food packaging (van der Walle *et al.,* 2001; Zinn *et al.,* 2001; Borah *et al.,* 2002; Luengo *et al.,* 2003).

It is well known that, our environment is contaminated with antibiotics and heavy metals originated from natural sources as well as direct or indirect human activities such as rapid industrialization, urbanization, and from human activities or anthropogenic activities (Krishnani and Ayyappan, 2006). Heavy metals used in industry and in domestic products, along with antibiotics used in agriculture, hospitals, animal husbandry, industry and prophylaxis, generating selective pressure that may leads to mutations for better survival under unfavorable conditions (Baquero *et al.,* 1998).

Interestingly, production of biopolymer during pilot studies or in industrial scale is greatly contaminated by environmental heavy metal especially in fermenters. De Lima *et al.* (1999) mentioned that PHA biopolymers production in industrial scale is strongly affected by heavy contamination from environment in the fermenter. Therefore, it is highly crucial to exploit the potent biopolymer producing strains regarding antibiotics and

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heavy metal resistance for controlling contamination before industrial production of the polymer (De Lima *et al.,* 1999; Rehman *et al.,* 2007; Razzaq *et al.,* 2010; Naheed *et al.,* 2011).

The purpose of this research is the study of factors controlling contaminants before industrial production of PHB biopolymer. Group of bacteria were isolated from soil or sewage samples in Eastern Province, Saudi Arabia, and screened for PHB production. Molecular characterization of the potent PHB-producing candidates was thoroughly investigated. Prominence was given to evaluation of biopolymer producing bacteria regarding sensitivity to antibiotics and resistance to heavy metals. Possible toxicity of PHB-producing bacteria to human health was also detected.

Materials and Methods

Sampling, Isolation and Screening for PHB Production

Collection of soil and sewage samples using sterile containers and bottles, from different areas (Dammam, Al-Khobar, Qateif and Al-Hassa) in Eastern Province, Saudi Arabia. Isolation of bacteria was carried out as following; soil or sewage sample was diluted in sterile distilled water and 0.1 mL was plated on nutrient agar (NA plates) with the following composition (g/L); Peptone 5; beef 3; NaCl 5 and agar 15. Separate colonies were isolated and further purified. The purified strains were subjected to screening for PHB production after cultivation on three different mineral salts production media: M1 or modified E2 medium (Berekaa and Al-Thawadi, 2012), M2 medium (g/L): ammonium sulfate; 2, KH_2PO_4; 6.67, $(NH_4)HPO_4$; 4, $MgSO_4.7H_2O$; 0.8, 5 mL traces element solution (HCl, 5; $FeSO_4.7H_2O$, 10; $CaCl_2$ 2; $MnSO_4.4H_2O$, 0.5; $ZnSO_4.7H_2O$, 2.2; $CuSO_4.5H_2O$, 1.9; $(NH_4)Mo_7O_{24}$ 0.1; $Na_2B_4O_7.10H_2O$, 0.02) and M3 medium (g/L): glucose, 10; ammonium sulfate; 2, KH_2PO_4; 0.5, $MgSO_4.7H_2O$; 0.2, NaCl; 0.1, peptone; 2.5 and yeast extract; 2.5) and incubation at 37°C. After sterilization of media, filter-sterilized stain was added (Sudan Black B 0.3 g in 70% Ethanol and Nile Red A stock solution 0.25 g/mL DMSO, use 20 µL to reach final concentration of 0.5 µg/mL).

Antibiotics and Heavy Metals Profiling

Potent PHB-producing strains were screened for antibiotic sensitivity through sensitivity test using Mueller Hinton agar medium as well as Agar Sensitivity Test medium (BioWorld, USA) (Bauer *et al.,* 1966). Eight different antibiotics used in this study namely; Ampicillin (10 µg), Erythromycin (5 µg), Tetracycline (30 µg), Kanamycin (30 µg), Streptomycin (10 µg), Ciprofloxacin (5 µg), Neomycin (30 µg), and Amoxicillin (30 µg). All antibiotics purchased from (BD BBLTM, USA), except Amoxicillin from (HARDY diagnostic, USA). For heavy metal resistance profiling different concentrations of the following metals were tested; $AgNO_3$, $ZnSO_4$, $CdCl_2$, $PbNO_3$ (stock solution: 0.4 g/mL); $K_2Cr_2O_7$ (stock solution: 0.2 g/mL) and $HgCl_2$ (stock solution: 0.1 g/mL).

Cultivation on Blood Agar

To test hemolytic activity, PHB-producing strains tested for hemolytic activity by cultivation on blood agar medium (Bioworld, USA).

Molecular Identification by 16S RNA Gene Analysis

Molecular identification of the bacterial strains was carried out by amplification of 16S RNA gene using set of primers listed in Table 1. After PCR amplification and subsequent sequencing, the DNA sequences of 16S RNA genes were analyzed by BLAST search on Genbank database of NCBI, NIH, USA.

Results

Screening and Molecular Identification

In a program for isolation of PHB-producing bacteria, a group of 120 bacterial strains were isolated from soil and sewage specimens from Eastern Province (Dammam, Al-Khobar, Qateif and Al-Hassa), Saudi Arabia. The strains were further purified and subjected to screening for PHB production by cultivation on the three PHA production media and subsequently tested by staining with Sudan Black B stain and Nile Red A. Positive strains showed blue-black colonies with Sudan black stain and red fluorescence after staining with Nile Red A and exposure to UV (Fig. 1).

Among screened bacteria, 16 strains showed clear positive results with both dyes, and thus chosen for further investigation. Molecular identification by 16S RNA gene analysis indicated that most of the isolated strains are bacilli belong mainly to members of *Bacillus cereus* group, *B. megaterium*, *B. flexus*, *B. endophyticus*, and *B. aryabhattai*. The potent PHB-producing candidates were given specific names, 16S DNA nucleotide sequence deposited in DNA Genbank and accession numbers are provided in Table 2.

Antibiotic Sensitivity and Heavy Metals Resistance Profiling

Eight different antibiotics were used to profile antibiotic sensitivity among PHB-producing candidates. Results indicated that all bacterial candidates are sensitive to most of tested antibiotics. However, bacterial strains *B. cereus* strain DS3-16 and *B. thuringiensis* strain DST1-33 recorded the highest resistance, especially to ampicillin, erythromycin and amoxicillin, while the least sensitive were *B. megaterium* strain DG8, strain DP7 and *B. pseudomycoides* strain DWST2-1 (Fig. 2a).

Table 1: List of primers used for amplification of 16S RNA gene from potent PHB producing strains

Label	Sequence	Reference
SFC1	5'-AGR GTT TGA TCM TGG CTC AG-3'	Hoseinabadi *et al.,* 2015
SRC1	5'-TAC GGY TAC CTT GTT AYG ACT T-3'	Hoseinabadi *et al.,* 2015
SRC2	5'-AAG GAG GTG ATC CAA CCG-3'	Logan *et al.,* 2000
SF1	5'-GAG TTT GAT CMT GGC TCA G-3'	El-Helow, 2001
SR2	5'- TAC GGY TAC CTT GTT ACG ACT T-3'	El-Helow, 2001

Table 2: DNA Sequence analysis of 16S RNA gene and hemolytic activity of PHB-producing candidates

Bacterial strain	Accession number	Hemolytic activity	Bacterial strain	Accession number	Hemolytic activity
B. thuringiensis DST1-33	KU199803	B	*B. megaterium* DP7	KU199812	Non
B. endophyticus DS43	KU199806	Non	*B. flexus* DS3-17	KU199813	Non
B. megaterium DPS6	KU199807	Non	*B. cereus* DS3-16	KU199814	β
B. axarquiensis DSG5	KU199808	α	*B. cereus* DS3-12	KU199815	β
B. sp. DS3-14	KU199805	Non	*B. pseudomycoides* DWST2-1	KU199816	β
B. megaterium DG8	KU199809	Non	*B. mycoides* DWST2-2	KU199817	Non
B. aryabhattai DBS10	KU199810	Non	*B. anthracis* DWST2-3	KU199818	Non
B. megaterium DT7	KU199811	Non	*B. megaterium* DPS8	KU199804	Non

On the other hand, profiling of heavy metal resistance among the group of PHB-producing bacteria indicated that *Bacillus* sp. strains DSG5, DPS6 followed by DSWT2-1, DS3-17 and DS3-12 showed highest resistance to Cr^{+6} (5 mg/mL) as well as Hg (62.5 µg/mL) and Ag (10 mg/mL), respectively while, other strains showed higher susceptibility to all metals including; Cd, Zn and Pb (10 mg/mL) (Fig. 2b).

Cultivation on Blood Agar

To test the possible toxicity of the PHB-producing candidates, hemolytic activity of the potent bacterial candidates was monitored by cultivation on Blood Agar medium. Results in Table 2 indicated that four isolates including; *B. cereus* strain DS3-16 and strain DS3-12, *B. thuringiensis* strain DST1-33 and *B. pseudomycoides* strain DWST2-1 showed β-hemolysis activity, while *B. axarquiensis* strain DSG5 showed α-hemolytic activity, reflecting their possible toxicity. However, *B. mycoides* strain DWST2-2, *B. aryabhattai* strain DBS10, *B. endophyticus* DS43 and *B. megaterium* strain DP7 are common soil non-pathogenic bacteria, recorded no hemolytic activity.

Discussion

A group of 120 bacterial strains isolated from soil and sewage specimens from Eastern Province, Saudi Arabia and screened for PHB production carried out by the use of several dyes. Sixteen strains showed blue-black colonies with Sudan black stain (non-specific for any lipid particles) and colonies with red fluorescence after staining with Nile Red and exposure to UV (specific for PHB). Generally, the dynamic status of our ecosystem contaminates soils with large diversity of PHB-producing bacteria (De Lima *et al.,* 1999). Chen reported the tremendous activity of bacteria in production of polyhydroxyalkanoates as a promising polymer for drug delivery and as biofuel (Chen, 2010). Chen *et al.* (2001) isolated 54 bacterial strains 97% were PHA producers as anticipated from Sudan black B and Nile Blue A staining. Molecular identification of the isolates indicated that all PHB-producing strains with highest homology to bacilli, especially members of *B. cereus* group namely; *B. pseudomycoides*, *B. mycoides*, *B. cereus* and *B. anthracis*, in addition to other bacilli namely; *B. megaterium*, *B. flexus*, *B. endophyticus* and *B. aryabhattai*. Interestingly, production of PHB by bacteria belong to this this group was recently reported (Valappil *et al.,* 2007; Aarthi and Ramana, 2011; Narayanan and Ramana, 2012). Narayanan and Ramana (2012) managed to isolate *B. mycoides* DFC1 from garden soil and optimized PHB production by central composite design.

Unlike PHB biopolymer produced from gram negative bacteria, biopolymer produced from gram positive bacteria especially bacilli is known to be free from toxic compounds especially outer membrane lipopolysaccharide (LPS) endotoxins that could induce severe immunogenic reaction (Chen *et al.,* 2005). Lee *et al.* (1999) made an attempt to remove endotoxins, while purifying the poly (3-hydroxybutyrate) from Gram-negative bacteria. Moreover, PHB-producing bacilli are capable of rapid growth on a variety of cost-effective substrates, while gram-negative bacteria require expensive substrates which should be structurally related substrate to produce the PHAs polymer. Therefore, large numbers of bacilli are used in PHAs production and recommended for biomedical applications (Chen *et al.,* 1991; Wu *et al.,* 2001; Yilmaz *et al.*, 2005; Valappil *et al.,* 2007; Singh *et al.,* 2009; Lopez-Cortes *et al.*, 2010; Mizuno *et al.,* 2010).

Our environment steadily gets overloaded with heavy metals and antibiotics contaminants from natural sources as well as human activities e.g., industrialization and urbanization activities (Krishnani and Ayyappan,

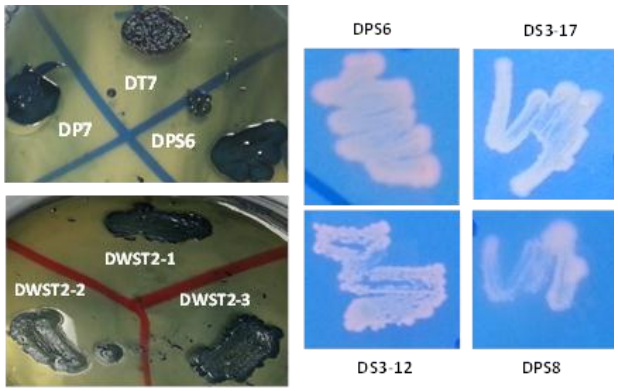

Fig. 1: Screening for PHB producing bacteria by staining with Nile Red A (right) and Sudan Black B (left)

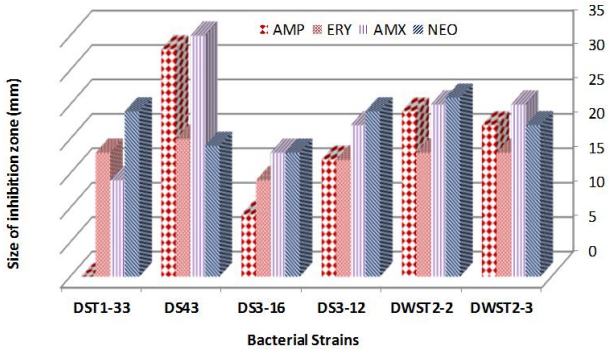

Fig. 2a: Antibiotic sensitivity profiling among the most resistant PHB-producing candidates

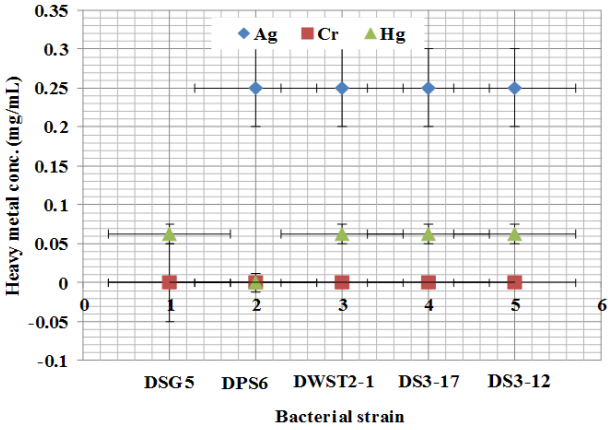

Fig. 2b: Heavy metal resistance among the most tolerant PHB-producing candidates

2006). In parallel, antibiotics misuse in different activities e.g. agriculture, hospitals, animal farming and industry, might pose serious pressure on microbes leading to mutations and other modifications. Therefore, it is recommended to explorer the PHB-producing strains regarding resistance to heavy metal and antibiotics for controlling contamination before industrial production of the biopolymer (De Lima *et al.,* 1999; Rehman *et al.,* 2007).

Except ampicillin, erythromycin, amoxicillin and neomycin, most of PHB-producing candidates showed higher susceptibility to antibiotics (Fig. 2) and heavy metals except Ag, Cr and Hg, respectively. Most of these bacterial candidates isolated from wild environment which might be the reason for limited number of resistance markers. In contrast, Naheed *et al.* (2011) showed that many of PHAs producing strains were resistant to multiple markers like commonly used antibiotics (penicillin and streptomycin) and heavy metals (Cu and Cd). The Cu^{+2} ion resistance (>6.5 mM) was more common among a population of PHB-producing bacteria (Chen *et al.,* 2001). Interestingly, some heavy metals, like copper II, trigger the biosynthesis and accumulation of PHB in some bacteria e.g., *Azospirillum brasilense* strains Sp7 (Kamnev *et al.,* 2012). Furthermore, in *Cupriavidus taiwanensis* EJ02, phylogenetically related to *Ralstonia eutropha*, the presence of heavy metals especially cadmium ions might be associated with its ability to accumulate PHB and to adapt to environmental stress (Chien *et al.*, 2014).

On the other hand, toxicity of producing organism might have negative impact on the process regarding biotechnological production of the biopolymer in large scale. In this study, four isolates including; *B. cereus* strain DS3-16 and strain DS3-12, *B. thuringiensis* strain DST1-33 and *B. pseudomycoides* strain DWST2-1 showed β-hemolysis activity, while *B. axarquiensis* strain DSG5 showed α-hemolytic activity, reflecting their possible toxicity. In fact, *B. cereus* group includes well known candidates showed powerful hemolytic activity and capable of causing food poisoning with diarrhea (Phelps and McKillip, 2002; Hendriksen *et al.,* 2006; Didelot *et al.,* 2009). On the other hand, *B. mycoides* strain DWST2-2, *B. aryabhattai* strain DBS10, *B. endophyticus* DS43 and *B. megaterium* strain DP7 are common soil non-pathogenic bacteria, recorded no hemolytic activity, thus can be advantageous in terms of PHB production. Indeed, immunological characteristics of these bacteria should be intensively investigated to ensure the safety of these bacterial candidates during biotechnological production of PHB in large scale.

Conclusion

Bacillus mycoides DWST2-2, *B. aryabhattai* DBS10, *B. endophyticus* DS43 and *B. megaterium* DP7 are common soil non-pathogenic bacteria, recording no hemolytic activity, thus can be used for biotechnological production of the PHB biopolymer.

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