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MOLECULAR CHARACTERIZATION AND UV IMPROVEMENT OF SOME BIOPLASTIC-PRODUCING BACTERIA ISOLATED FROM PLANTS IN TAIF CITY, SAUDI ARABIA

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ABSTRACT

In this study, 98 polyhydroxybutyrate (PHB) accumulating bacterial isolates were isolated from rhizosphere of 5 different plants in Taif City, Saudi Arabia. PHB production was tested by Sudan Black B staining on N-free agar and nutrient agar media as a primary qualitative screening method followed by Nile Blue A staining. The results showed that 18 out of 98 isolates were positive for Sudan black B on nutrient agar medium, and 10 out of 18 isolates were positive on N-free agar medium. Seventeen out of 18 PHB producing strains confirmed by Sudan black B staining showed Pink/orange florescence under UV light when stained with Nile Blue A on carbon rich nutrient agar medium. Three isolates (HO1, HO12 and HO17) showed high production of PHB on three different media (nutrient, carbon rich nutrient and N-free agar). The PHB isolates were further characterized by biochemical testing and 16S rRNA sequencing. Partial sequencing of 16 S rRNA was performed in 11 positive PHB producing isolates (HO1, HO2, HO8, HO9, HO10, HO11, HO12, HO3, HO4, HO7, and HO5). Sequences were deposited in the GenBank with accession numbers KY313628.1, KY313629.1, KY313635.1, KY313636, KY313637, KY313638, KY313639 (*Acinetobacter haemolyticus*), KY313630.1, KY313631.1, KY313634.1 (*Acinetobacter lwoffiei*) and KY313632.1 (*Acinetobacter baumannii*). A promising bacterial isolate, *Acinetobacter haemolyticus* (HO1), was found to produce 47% PHB per dry weight in 72 hr. incubation. This isolate was selected for PHB yield improvement by UV irradiation. The PHB-producing ability of supposed mutant strains of *A. haemolyticus* (HO1) was found to increase substantially in the range of 51% to 67.05%. Complete cell proteins were isolated from 7 mutants and were compared with parental strain. It was found that the protein profiles of the high PHB-yield mutant strains were generally differed from the protein profiles of parental strain. Plasmid profile results showed that five strains (HO1, HO8, HO4, HO11 and HO5) and seven UV mutants (M2.10, M36.10, M2.5, M3.5, M6.5 and M61.5) harbored one plasmid (25 Kb) except (M3.10) mutant strain which has lost its plasmid on UV irradiation.

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Introduction

Decomposition of plastics and synthetic polymers is very challenging owing to their origin to petrochemicals. Accumulation of synthetic polymers leads to environmental pollution, and likewise is dangerous to a number of animal species [1, 2]. Every year the amount of plastic waste increases. The disposal of plastic waste has become a major environmental problem world-wide. Studies have reported that 5x107 tons of post-consumer plastic waste is produced only by USA, Europe and Japan [3]. Plastic waste in Saudi Arabia resembles 15% in the composition of domestic municipality waste [4].

Plastics derived from renewable biomass sources such as vegetable oil, corn starch, and pea starch are called bioplastic. The nuclear molecule involved in synthesis of bioplastic is polyhydroxybutyrate (PHB). Some bacteria produce PHB by fermentation, and use it as a food storage molecule [5]. PHB (-[CH(CH3)CH2COO]n-) is an eco-friendly polymer, because

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it is fully biodegradable in short time. Physical properties of PHB are similar to polypropylene including its high crystallinity; therefore, it has a high potential for use in industry [6].PHB can be recycled by microorganism to carbon dioxide and water. Different soil bacteria have the capacity to produce and degrade PHB [7, 8].*B. megaterium* and *Azospirillum* spp have the ability to produce PHB. Bacillus subtilis was identified by means of 16S rRNA analysis and was initially isolated from sewage sample. *B. megaterium* and *Azospirillum* spp were isolated from the soil sample [9]. PHB can be recycled. This property makes it a suitable material to replace synthetic plastics in many applications [10]. It has biomedical applications as drug delivery and medical implants. The researchers working on biodegradable plastic have a focus on the isolation of PHB-producing microorganisms and efficiency improvement of those organisms in terms of PHB synthesis using less expensive substrates [11]. Use of UV-radiations to obtain genetically modified PHB-producing microorganism in order to increase the PHB yield showed promising results [12, 13].

The present study was designed with the aim to isolate PHB producing bacteria from different rhizospheric areas of 5 plants in Taif city. PHB producing isolates were screened to detect high PHB yielding isolates using biochemical and molecular approaches. UV radiations were used to obtain genetic improvement in the best poly-β-hydroxybutyrate (PHB) granules producing bacteria followed by comparison of the PHB production capacities using total protein profiles.

Materials and Methods

Media

Nutrient agar medium (NA) and Ashby or N-free agar medium were prepared according to the methods described by [14, 15], respectively.

Collection of samples

Soil samples were collected from different plantation in Taif City, Saudi Arabia. The samples were taken from rhizosphere region of 5 plants (*Phaseolus vulgaris*, *Cucurbita pepo*, *Brassica oleracea*, *Eruca sativa* and *Anethum graveolens*) along with three samples which were taken from soil.

Isolation of bacteria

One gram of the soil was taken, and 9 ml of sterile distilled water was added. A serial dilution up to $10^{-7}$was prepared. One hundred µl from suspension of three dilutions ($10^{-3,}$ 10^{-5} and10^{-7}) were spread on nutrient agar plates followed by incubation at 30º C for 24 hr.

Detection of PHB producers using Sudan black B and Nile blue A staining

The bacterial isolates were grown on nutrient and N-free agar media and examined for polyhydroxybutyrate (PHB) accumulation by staining with Sudan black B. All plates were incubated at 30°C for 72 hrs. After incubation, the plates were flooded with an ethanol solution containing 70% ethanol and 0.3% Sudan black B for 20 mins followed by washing the plates with 96% ethanol to remove the excess stain. Isolates showing dark blue color were taken as PHB positive [16]. Sudan black B positive isolates were further screened with Nile blue A staining on carbon rich nutrient agar medium containing 0.0005 g Nile blue. Colonies containing PHB accumulating strains were stained with Nile blue A showed bright orange or pink fluorescence on irradiation with UV light. The isolates which showed orange or pink fluorescence on irradiation with UV light were considered PHB positive [17].

Moreover, a slide method was also used to detect PHB producers. Spreading bacterial colony on a clean glass slide was fixed by heating, followed by addition of few drops of Sudan black B solution (0.3% in 70% ethanol) in order to stain them. After 20 mins of incubation, slide was immersed several time in xylene solution and blot dried with absorbent paper, followed by addition of 0.5 % Suffranin solution. The slide was washed with tap water, dried and observed under oil immersion microscope as described elsewhere [9].

Identification of the isolated bacteria's Biochemical characteristics

Biochemical identification of 18PHB producing bacteria was performed using Enterosystem 18R kit according to the manufacturer instructions (Liofilchem/Italy). These tests are simple to use, cost effective and efficient in biochemical identification of gram-negative and oxidase-negative EnteroPCR bacteria.

Molecular identification of PHB producers by 16S rRNA

Genomic DNA was extracted from 11PHB producing bacteria, grown on nutrient broth medium, using GenElute™ bacterial genomic DNA kit (Sigma-Aldrich Co. LLC). The 16S rRNA genes of the isolated bacteria were amplified using forward 5'-AGAGTTTGATC(A/C)TGGCTCAG-3' and reverse primers 5'-TACGG (C/T)TACCTTGTTACGACTT-3'. PCR were performed with thermal cycler in a final volume of 50 µl. DNA sequences were obtained using a 3130 XL Genetic Analyzer (Applied Biosystems, Hitachi, Japan). Following reaction conditions were used; 95°C for 4min, 30 cycles of 94°C for 1 min at, 50-59°C for 1min and 72°C for 2 mins followed by an additional cycle of 10 min at 72°C. The annealing temperature was set at 52-58°C.

The 16S rDNA sequences which have been determined in this study were deposited in NCBI database (www.ncbi.nlm.nih.gov). Sequence analysis was performed using a reference sequencer deposited by [18].

Antibiotic Test

Disk diffusion susceptibility test using 8 different antibiotics [streptomycin-S (10 mg), ampicillin/sulbactam-SAM (20 µg), levofloxacin-LEF (5 µg), tigecycline-TGC (15 µg), tobramycin-TMN (10 µg), Vancomycin-Va (30 µg), clindemycin-CM (2 µg) and metronidazole-MET (50 mg)] were used to determine the resistance or sensitivity of the *A. baumannii* (HO5), *A. haemolyticus* (HO1, HO2, HO10, HO11, HO8, HO12, HO13 and HO15) and *A. lwoffi* (HO4, HO3 and HO7) strains. Briefly, the bacterial strains were grown in nutrient broth for 24 hrs at 37 ºC. One microliter of the over-night grown culture was spread on the surface of Mueller-Hinton in the presence of various antibiotics impregnated filter paper disks as described elsewhere [19].

Genetic improvement of *A. haemolyticus* (HO1) isolate for PHB production

Ultraviolet (UV) irradiation was used for improvement of PHB production from *Acinetobacter haemolyticus* (HO1) strain using the protocol described by [20] Bacterial cells were grown in nutrient broth medium. One ml of *A. haemolyticus* (HO1) suspension was placed into Petri dishes at a distance of 11.5 cm followed by UV irradiation for 5 and10 mins at 254 nm. The plates were then kept in dark box for 2hrs. 1 ml of *A. haemolyticus* (HO1) culture was spread on NA plates and incubated for 24 hrs at 30°C. Colonies developed after incubation were counted and transplanted to slants in order to estimate the survival percentages for each treatment as described in [21].

PHB extraction from *A. haemolyticus* (HO1) parental and mutant strains

PHB was extracted from *Acinetobacter haemolyticus* (HO) parental and mutant strains grown on N-free broth medium after 72 hr incubation at 30 °C. Briefly, 200 ml of culture was centrifuged at 4500 rpm for 30 mins. The supernatant was thrown out. The pellet was treated with 10 ml of 6% sodium hypochlorite solution and incubated at 37 °C for 1.5 hrs. The mixture was then centrifuged at 4500 rpm for 20 mins followed by washing with distilled water, acetone, and methanol sequentially. The pellet was re-suspended in 10 ml of chloroform, and chloroform was evaporated by pouring the solution on sterile tray and kept in hot air oven at 40 °C [22].

Estimation of cell dry weight

Cell dry weight was determined by centrifugation of the 200 ml culture incubated for 72 hrs at 30 °C on Ashby medium. Supernatant was discarded and the pellet was washed with distilled water. The washed pellet was resuspended in distilled water and then transferred to filter paper and placed in an oven at 80 °C for 7 hrs followed by dry weight measurement [23].

Determination of Plasmid pattern

Plasmid pattern was determined using plasmid isolation kit (Promega plasmid miniprep kit). Plasmid was isolated from five strains (*A. baumannii* HO5, *A. haemolyticus* (HO1, HO8 and HO11) and *A. lwoffi* HO4 and seven mutant strains of *A. haemolyticus* (HO1) grown in nutrient broth after UV exposure.

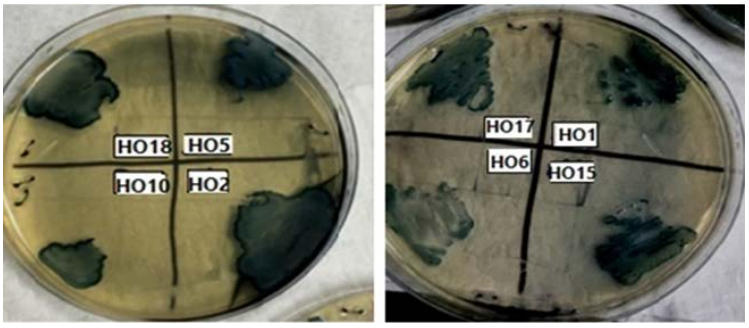
Results

Sample collection and bacterial isolation

In this study, PHB-accumulating bacterial strains were isolated from rhizosphere of 5 different plants in Taif city of Saudi Arabia. In total 98 bacterial isolates were collected including 31 isolates from plants; *Phaseolus vulgaris*, 17 isolates from *Cucurbita pepo*, 10 isolates from *Brassica oleracea*, 8 isolates from *Eruca sativa*, 22 isolates from *Anethum graveolens* and 10 isolates from soil were isolated and screened for PHB production.

Detection of PHB producers by Sudan black B and Nile blue Astaining

Sudan black B staining was used for initial screening of 98 isolates grown on nutrient agar medium. Based on Sudan black B staining 18 isolates, PHB positive isolates were detected (5 isolates from *Phaseolus vulgaris*, 2 isolates from *Brassica oleracea*, 2 isolates from *Anethum graveolens*, 1 isolate from *Eruca sativa*, 1 isolate *Cucurbita pepo* and 7 isolates from soil) (Table 1) and (Fig. 1). N-free agar medium was used for further screening, and the results showed that 10 of 18 isolates showed black-blue coloration when stained with Sudan black B (Fig. 2) including 4 high PHB producing isolates (HO1, HO12, HO15 and HO17). 18 Sudan black B positive isolates further screened with Nile blue on carbon rich nutrient agar medium (0.0005 g Nile blue, supplemented with 1% glucose) indicated that 17 isolates showed Pink/orange florescence under UV light (Fig. 3). Three isolates (HO1, HO12 and HO17) showed high production of PHB on three media (nutrient, carbon rich nutrient and N-free agar media).



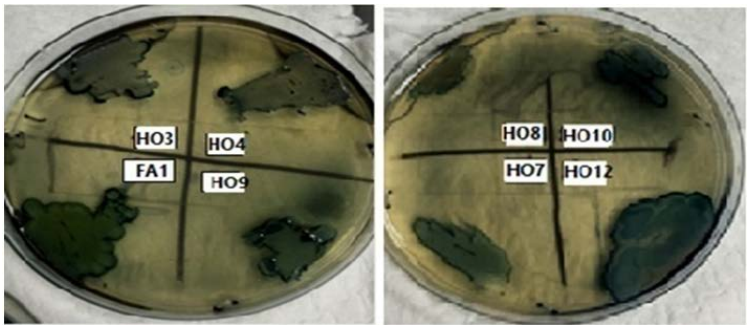


Fig. 1.PHB producing isolate screening by Sudan black B staining on nutrient agar plates, all dark blue or black colored colonies stained cultures were positive for PHB production.

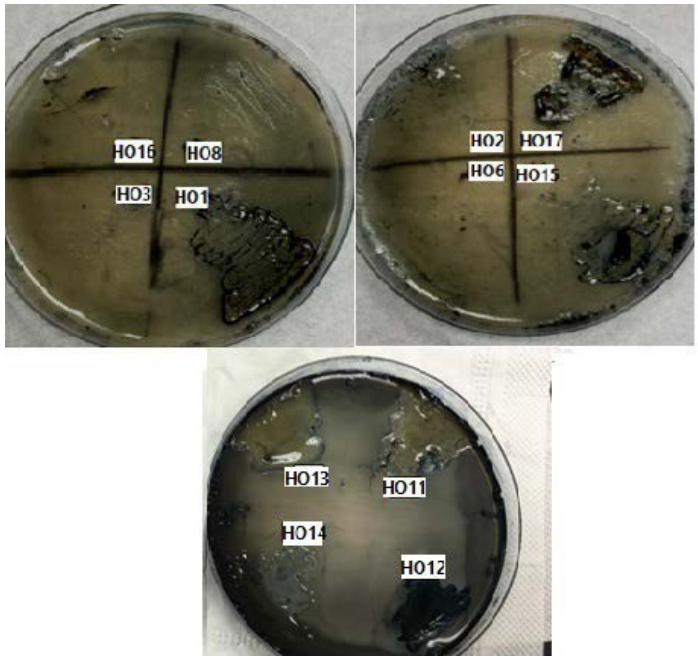


Fig. 2. Screening of PHB production isolates by Sudan black B staining on N-free agar medium

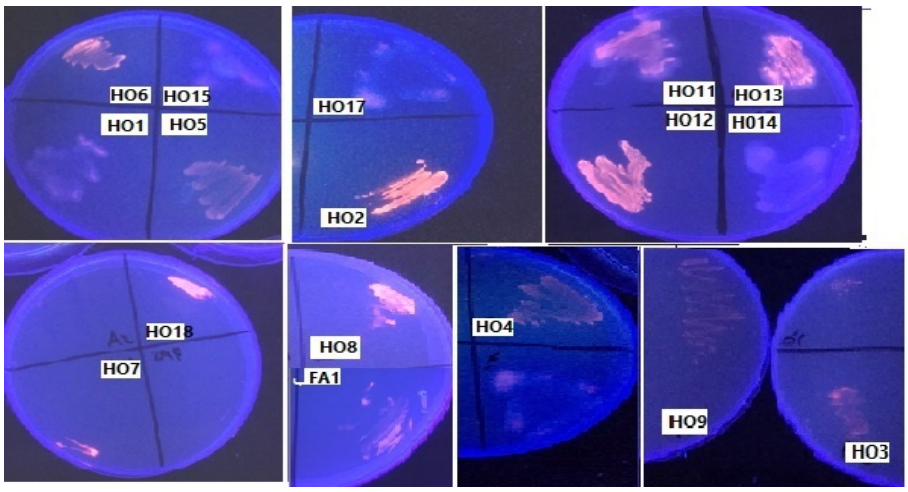


Fig. 3. Pink/orange florescence under UV light by PHB producer isolates HO1, HO2, HO3, HO4, HO5, HO6, HO7, HO8, HO9, HO11, HO12, HO13, HO14, HO15, HO17, HO18 and FA1

Table 1. Screening of PHB producers by Sudan black B and Nile blue A staining on three media

Source of isolates	Type of isolate	Staining Method		
		Sudan black		Nile Blue
		Nutrient agar medium	N-free agar medium	Nutrient agar medium + 1% glucose
Rhizosphere of Beans	HO1	++	+++	++
Soil sample	HO17	++	+++	++
Soil sample	HO12	++	++	++
Rhizosphere of *Cucurbita pepo*	HO2	++	+	++
Rhizosphere of Cauliflower	HO15	++	++	++
Rhizosphere of Cauliflower	HO3	++	_	++
Rhizosphere of *Anethum graveolens*	HO4	++	+	++
RhizosphereofBeans	FA1	+	_	++
Sample from soil	HO5	++	+	++
Sample from soil	HO6	++	_	++
Rhizosphere of Beans	HO18	++	_	++
Rhizosphere of Beans	HO7	++	_	++
Rhizosphere of Beans	HO8	++	_	++
Rhizosphere of *Anethum graveolens*	HO9	+	_	++
Rhizosphere of *Eruca sativa*	HO10	++	_	_
Sample from soil	HO11	+	+	++
Sample from soil	HO13	+	+	++
Sample from soil	HO14	+	+	++
Sample from soil	HO16	_	_	_

Detection of PHB producers by Sudan black B under light microscope

Sudan black B and Nile blue A positive PHB producers (HO1, HO17 and HO12) were further examined under light microscope. Dark black color PHB granules were observed inside the cell (Fig. 4).

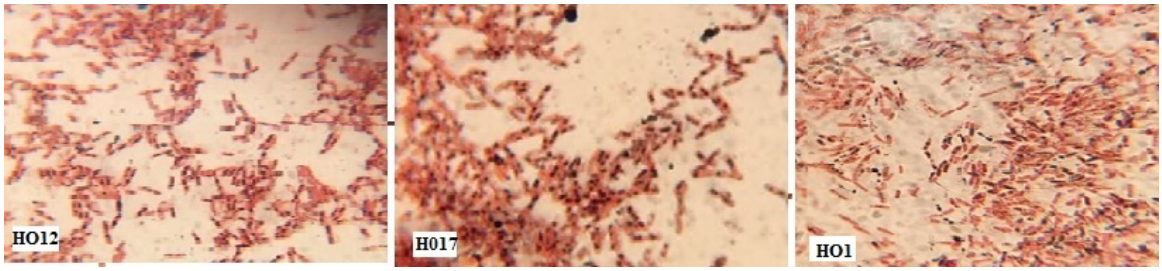


Fig. 4. Dark black PHB granules observed using light microscope in isolates HO1, HO17 and HO12

Biochemical identification of PHB producing bacteria

Biochemical characteristics of 17 potent PHB producing strains are shown in Table 2. Among all the positive PHB producing strains, 13 isolates (HO1, HO2, HO8, HO9, HO10, HO11, HO12, HO13, HO14, HO15, HO17, HO18 and HO19) were identified as *Acinetobacter haemolyticus*. Three isolates (HO3, HO4 and HO7) were identified as *Acinetobacter lwoffii* and 1 isolate (HO5) as *Acinetobacter baumannii* (Table 2, 3).

Table 2. Biochemical characteristics of bacteria screened for PHB production

Test / isolates	ONPG	LDC	ODC	ADC	PD	CIT	UR	H2S	MLN	VP	IND	GLU	MAN	INO	SOR	SAC	ARA	RAF
HO3	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
HO16	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-
HO5	-	+	+	+	-	+	+	-	+	-	-	+	-	-	-	-	+	-
HO17	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HO2	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
HO7	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-
HO4	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
HO18	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
HO8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
HO1	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-
HO15	-	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-
HO14	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
HO11	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
HO13	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
HO12	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HO10	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
HO19	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	+	-
HO9	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-

Table 3. Types of bacterial isolates after biochemical identification

Isolates	Strains	Isolates	Strains
HO1	*Acinetobacter haemolyticus*	HO3	*Acinetobacter lwoffii*
HO2	*Acinetobacter haemolyticus*	HO4	*Acinetobacter lwoffii*
HO8	*Acinetobacter haemolyticus*	HO7	*Acinetobacter lwoffii*
HO9	*Acinetobacter haemolyticus*	HO5	*Acinetobacter baumannii*
HO10	*Acinetobacter haemolyticus*	HO13	*Acinetobacter haemolyticus*
HO11	*Acinetobacter haemolyticus*	HO16	*Morganella morganii*
HO12	*Acinetobacter haemolyticus*	HO17	*Acinetobacter haemolyticus*
HO19	*Acinetobacter haemolyticus*	H018	*Acinetobacter haemolyticus*
HO14	*Acinetobacter haemolyticus*	HO15	*Acinetobacter haemolyticus*

Molecular identification of the isolated bacteria

The type of 11 selected bacterial isolates (HO1, HO2, HO8, HO9, HO10, HO11, HO12, HO3, HO4, HO7 and HO5) were further confirmed by using 16S rRNA gene sequencing. Sequences were submitted to GenBank (www.ncbi.nlm.nih.gov) under accession numbers KY313628.1, KY313629.1, KY313635.1, KY313636.1, KY313637.1, KY313638.1, KY313639.1, KY313630.1, KY313631.1, KY313634.1 and KY313632.1 respectively. Table 4 and Fig. 5 show that three strains HO3, HO4 and HO7 belong to *Acinetobacter lwoffi*, HO5 strain belongs to *Acinetobacter baumannii* and HO1, HO2, HO8, HO9, HO10, HO11 and HO12 strains belong to *Acinetobacter haemolyticus*. Dendrogram demonstrating the relationship among the 11 strains based on data recorded from 16S rRNA using T-Coffee program (Fig. 5).

Phylogenetic analysis revealed that *Acinetobacter haemolyticus* HO9 strain obtained from the anethrhizosphere formed a separate cluster away from the known Acinetobacter-type strains (Fig. 5). It was also seen from the phylogenetic analysis that strain HO11 identified as *Acinetobacter haemolyticus*, clades with *A. lwoffi*; indicating that this strain is probably a new species of the genus Acinetobacter. The strains HO2, and HO10 the type strains of *Acinetobacter haemolyticus* from different plants fell in a single clade whereas, HO8 formed a separate clade, indicating the presence of different phylotypes in the soil samples.

Table 4. Results of Blast search for DNA sequence in NCBI Genbank

Isolates	Accession No.	Query	Total score	Query cover%	E-value	Identity%
Acinetobacter haemolyticus	KY313628.1	HO1	1583	99%	0.0	99%
	KY313629.1	HO2	1576	100%	0.0	99%
	KY313635.1	HO8	1591	99%	0.0	99%
	KY313636.1	HO9	1439	100%	0.0	99%
	KY313637.1	HO10	1459	99%	0.0	99%
	KY313638.1	HO11	1452	99%	0.0	99%
	KY313639.1	HO12	1218	100%	0.0	99%
Acinetobacter lwoffii	KY313630.1	HO3	1251	100%	0.0	99%
	KY313631.1	HO4	1138	100%	0.0	99%
	KY313634.1	HO7	1568	100%	0.0	99%
Acinetobacterbaumannii	KY313632.1	HO5	1315	100%	0.0	99%

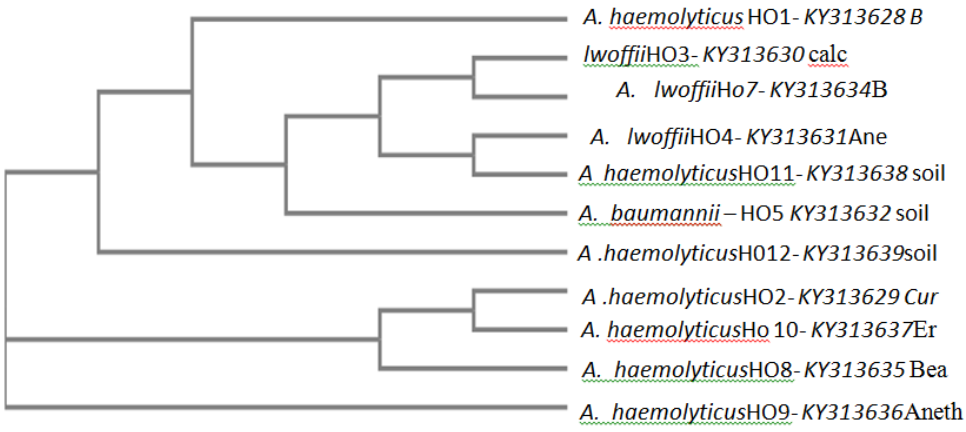


Fig. 5. Phylogenetic tree of bacterial strains

Antibiotic Test

Data in Table 5 show that all strains are resistant to clindemycin (CM), ampicillin / sulbactam (SAM) and metronidazole (MET). Six strains are sensitive to levofloxacin (LEV) including *A.haemolyticus* (HO11, HO13, HO15, HO8), *A. lwoffii* (HO7 and HO4), except one strain of *A. haemolyticus* (HO10) and 2 strains of *A .haemolyticus* (HO10, HO13). 10 strains are resistant to vancomycin (AV). 6 strains were resistant to streptomycin (S) including *A. haemolyticus* (HO13, HO10, HO12), *A. lwoffii* (HO4, HO3) and *A. baumannii* (HO5). Eight strains were found resistant to tobramycin (TMN).

Table 5. Sensitivity (S) and resistant (R) of some strains to antibiotics by disc diffusion method

Strains	Bacterial strains	Diameter of inhibition zone (in mm)							
		LEV	**TGC**	AV	**S**	**TMN**	**CM**	**MET**	**SAM**
		SIR							
1	*A.haemolyticus* HO11	23 (S)	19 (I)	12 (R)	16 (I)	13 (R)	15 (R)	0 (R)	6 (R)
2	*A. lwoffii* HO7	23 (S)	17 (I)	16 (I)	19 (I)	18 (I)	15 (R)	7 (R)	7 (R)
3	*A.haemolyticus* HO13	25 (S)	14 (R)	0 (R)	15 (R)	0 (R)	11 (R)	0 (R)	0 (R)
4	*A. baumannii* HO5	20 (I)	18 (I)	0 (R)	12 (R)	13 (R)	8 (R)	0 (R)	9 (R)
5	*A. lwoffii* HO4	23 (S)	16 (I)	9 (R)	15 (R)	10 (R)	12 (R)	7 (R)	7 (R)
6	*A.haemolyticus* HO10	15 (R)	13 (R)	16 (I)	7 (R)	11 (R)	13 (R)	6 (R)	6 (R)
7	*A. lwoffii* HO3	20 (I)	16 (I)	11 (R)	15 (R)	12 (R)	13 (R)	0 (R)	0 (R)
8	*A.haemolyticus* HO1	20 (I)	16 (I)	14 (R)	17 (I)	17 (I)	14 (R)	0 (R)	0 (R)

9	*A.haemolyticus* HO15	22 (S)	16 (I)	15 (R)	17 (I)	17 (I)	14 (R)	6 (R)	7 (R)
10	*A.haemolyticus* HO2	20 (I)	16 (I)	14 (R)	19 (I)	19 (I)	14 (R)	0 (R)	7 (R)
11	*A.haemolyticus* HO8	23 (S)	16 (I)	0 (R)	17 (I)	10 (R)	0 (R)	0 (R)	0 (R)
12	*A.haemolyticus* HO12	20 (I)	18 (I)	0 (R)	15 (R)	10 (R)	13 (R)	7 (R)	8 (R)
Anitbiotic resistance rate		50	100	100	100	100	100	100	100

Inhibition zone diameters were measured inclusive of the diameter of the discs. (s) Sensitive (≥ 21 mm); (I) Intermediate (16-20 mm); Resistant (≤ 15 mm). streptomycen (S), ampicillin / sulbactam (SAM), levofloxacin (LEV), tigecycline (TGC), tobramycin (TMN), vancomycin (AV), clindemycin (CM) and metronidazole (MET)

UV mutagenesis for *A. haemolyticus* (HO1) at 11.5 cm distance

In order to improve PHB production, the highest PHB producing *A. haemolyticus* (HO1) strain, as determined by all three media, was selected for UV mutagenesis. *A. haemolyticus* (HO1) strain was exposed to UV radiations at 11.5 cm distance for two times, 5 and 10 mins, respectively. Mutants were isolated and compared with wild type for production of PHB. Results in Table 6 indicate that survival rate for *A. haemolyticus* (HO1) strain exposed to UV for 5 mins was 0.03 %, and the survival rate was 0.01 % in strain exposed to UV for 10 mins.

Table 6. Survival of A. haemolyticus (HO1) after UV exposure at a distance of 11.5 cm

Strain	Exposure time (min.)	No. of colonies germinated after UV exposure at 254 nm	Survival rate (%)
A. haemolyticus (HO1)	0	16×10^4	100
	5	59	0.03
	10	22	0.01

Quantification of PHB for A. haemolyticus (HO1) and UV mutant strains

The powdered sample was extracted by sodium hypochlorite method, and further quantified to know the amount of PHB extracted from *A. haemolyticus* HO1 strain before and after conducting UV mutagenesis experiments. Table 7 showed that the percentage of PHB per dry weight of wild strain is 47.36 %. UV induced mutation increased PHB productivity in five variants (Mut 2.10, Mut 3.10, Mut 2.5, Mut 3.5 and Mut 61.5) to 67%, 65.4%, 57.3%, 54% and 51.2% respectively in comparison to wild organism.

Table 7: PHB accumulation of *A. haemolyticus* (HO1) parental and UV mutants

No. of mutants	Cell dry weight 200 ml (g)	PHB production/ 200 ml (g)	% PHB
wilde type	0.76	0.36	47.36%
Mut 2.10	0.85	0.57	67.05%
Mut 3.10	0.81	0.53	65.4%
Mut 36.10	0.6	0.27	45%
Mut 2.5	0.82	0.47	57.3%
Mut 3.5	0.77	0.42	54%
Mut 61.5	0.78	0.40	51.2 %
Mut 6.5	0.51	0.21	41.17%

SDS-PAGE to determine total cell proteins

Total cell protein profile analysis (SDS-PAGE) of *A. haemolyticus* (HO1) parental and mutant strains was performed to determine distances/similarities **(Table 8**). The results in table (4.10) showed that the similarities of Mut 2.5, Mut 36.10, Mut 61.5, Mut 3.5 Mut 2.10, Mut 3.10 were 94.00%, 93.00%, 90.00%, 87.00%, 84.00% and 56.00% respectively. Figure (4.9) showed appearance of 11 KD band in 5 mutant strains (Mut 2.10, Mut 36.10, Mut 61.5, Mut 3.5 and Mut 2.5) as shown in lane (3, 5, 6, 7 and 8) respectively. The dendrogram demonstrating the relationship among the six mutant and parent strains is based on data recorded from protein patterns (Fig.6).

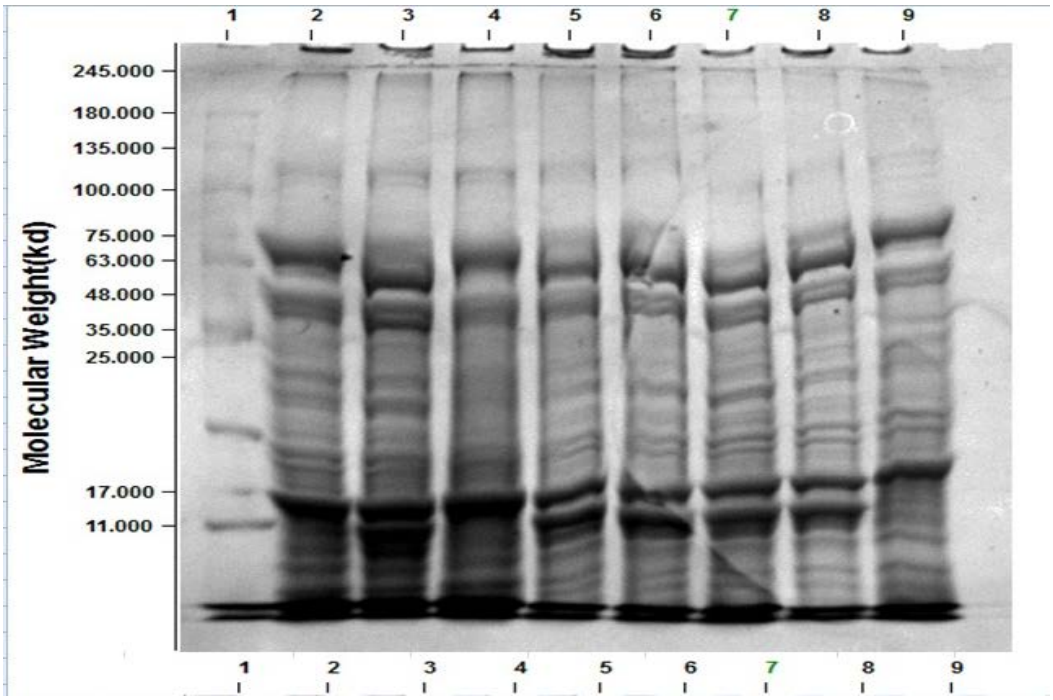


Fig. 6. Protein profile of parental and mutant strains. Lane 1; molecular weight markers, Lane3; mut 2.10, Lane4; mut 3.10, Lane5; mut 36.10, Lane6; mut 6.5, Lane7; mut3.5, Lane 8; mut2.5, Lane 9; parental strain

Table 8. Distance and similarities of parental and mutant strains

Mutant strains	Exposure time	Similarities	Distance
Mut 2.5	5	94.00%	6.00%
Mut 36.10	10	93.00%	7.00%
Mut 61.5	5	90.00%	10.00%
Mut 3.5	5	87.00%	13.00%
Mut 2.10	10	84.00%	16.00%
Mut 3.10	10	56.00%	44.00%

Plasmid Profile

Plasmids were isolated from *A. baumannii* (HO5), *A.haemolyticus* (HO1, HO8 and HO11) and *A. lwoffi* (HO4) strains. According to the SDS-PAGE electrophoresis data, all strains had one plasmid. Also, plasmids isolated from both *A.haemolyticus* (HO1) parental and mutant strains with different UV exposure time were subjected to electrophoresis. Results indicated that all mutant strains (M2.10, M36.10, M2.5, M3.5, M6.5 and M61.5) carry one plasmid (25 Kb) except 1 strain (M3.10) which has lost the plasmid (Fig. 7).

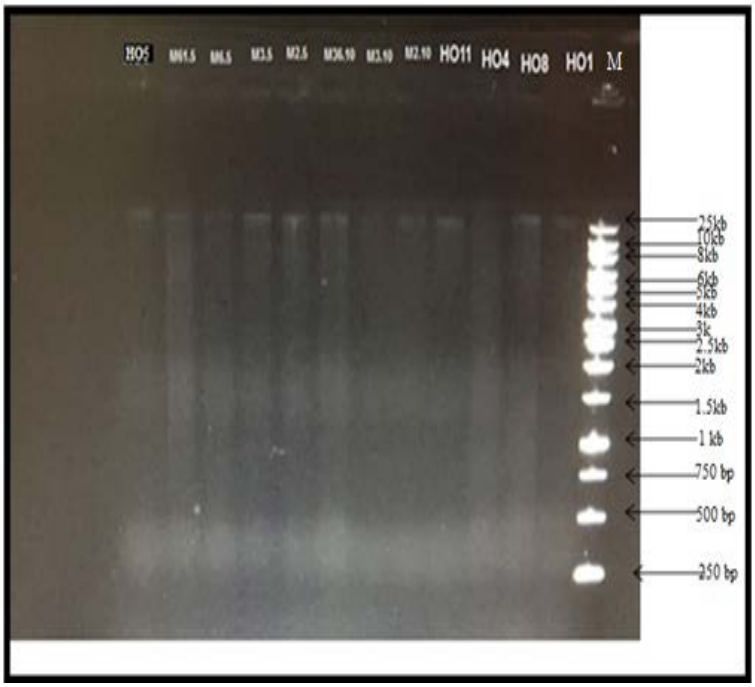


Fig. 7. Agarose gel electrophoresis of plasmid DNA Lane 1; *A. haemolyticus* HO1, Lane 2; *A. haemolyticus* HO8, Lane 3; A. lwoffi HO4, Lane 4; *A. haemolyticus* HO11, Lane 12; *A. baumannii* HO5, Lane 5; M2.10 after 10 min UV exposure, Lane 6; M3.10, Lane 7; M36.10, Lane 8; M2.5, Lane 9; M3.5, Lane10; M6.5, Lane11; M61.5, and Lane M; DNA ladder ranged from 250bp to 25kb.

Discussion and Conclusion

Bacterial colonies isolated from rhizospheric region of 5 different crops, commonly grown in Saudi Arabia, and soil samples were purified and cultured on nutrient agar medium. A total of 98 representative bacterial colonies (31from rhizospheric area of *Phaseolus vulgaris*, 17 from *Cucurbita pepo*; 10 from *Brassica oleracea*, 8 from *Eruca sativa* and 22 from *Anethum graveolen*, 10 from soil) were initially screened for PHB production using Sudan Black dye. Results of screening of 98 isolates by Sudan black B staining on nutrient media revealed that 18 isolates (5 from *Phaseolus vulgaris*, 2 from *Brassica oleracea*,2 from *Anethum graveolens*,1 from *Eruca sativa*, 1from *Cucurbita pepo* and 7 from soil) were positive for Sudan black B on nutrient agar medium. Further screening on N-free agar medium showed that 10 of 18 isolates showed black-blue coloration when stained with Sudan black B including four high PHB producing isolates (HO1, HO12, HO15 and HO17). The 18 Sudan black B positive isolates were also stained with Nile blue A on carbon rich nutrient agar medium (0.0005 g Nile blue, supplemented with 1% glucose) indicated that 17 isolates show Pink/orange florescence under UV light. Further analysis showed that the three isolates (HO1, HO12 and HO17) showed high production of PHB on three media (nutrient, carbon rich nutrient and N-free agar).

Biochemical screening revealed that the 17 isolates include 3 strains of *Acinetobacter lwoffi* (HO3, HO4 and HO7), 13 strains of *Acinetobacter haemolyticus* (HO1, HO2, HO8, HO9, HO10, HO11, HO19, HO14, HO13, HO17, HO15, HO18 and HO12) and 1 strain of *Acinetobacter baumannii* (HO5). 11 selected bacterial isolates (HO1, HO2, HO8, HO9, HO10, HO11, HO12, HO3, HO4, HO7 and HO5) were further confirmed by using 16S rRNA gene sequencing. The 16S rRNA sequences were submitted in GenBank with the following accession numbers KY313628.1, KY313629.1, KY313635.1, KY313636.1, KY313637.1, KY313638.1, KY313639.1, KY313630.1, KY313631.1, KY313634.1 and KY313632.1 respectively. Dendrogram demonstrating the relationship among the 11 strains based on data recorded from 16S rRNA using T-Coffee. Phylogenetic analysis revealed that *Acinetobacter haemolyticus* HO9 strain obtained from the *Anethum graveolens* rhizosphere formed a separate cluster away from the known Acinetobacter-type strains. It was also seen from the phylogenetic analysis that strain HO11 identified as *Acinetobacter haemolyticus*, clade with *A. lwoffi*; indicating that probably this strain is a new species of the genus Acinetobacter. The strains HO2, and HO10 the type strain of *Acinetobacter haemolyticus* from different plants fell in a single clade whereas HO8 formed separate clade, indicating the presence of different phylotypes in the soil samples. Twelve strains including *A. baumannii* (HO5), *A.haemolyticus* (HO1, HO2, HO8, HO10, HO11, HO12, HO13 and HO15) and A. lwoffi (HO3, HO4 and HO7) were tested for resistance against 8 antibiotics. Results showed that all strains were resistant to clindemycin (CM), ampicillin/sulbactam (SAM) and metronidazole (MET), 6 strains were sensitive to levofloxacin (LEV) including *A.haemolyticus* (HO11, HO13, HO15, HO8), *A. lwoffii* HO7 and HO4 except *A .haemolyticus* (HO10) strain. *A. haemolyticus* (HO10, HO13) strain were resistant to vancomycin (AV). 6 strains were resistant to streptomycin (S) including *A. haemolyticus* (HO13, HO10, and HO12), *A. lwoffii* (HO4, HO3) and

A. baumannii (HO5). 8 strains were found resistant to tobramycin (TMN). Previous studies have shown that *A.baumannii* showed high resistance to the evaluated antibiotics except ampicillin-sulbactam [24]. Similarly, sensitivity to ampicillin/sulbactam alone or in combination with rifampicin has also been reported [25]. Moreover, A. haemolyticuswas found sensitive to antibiotics tobramycin, lomefloxacin, but resistant to streptomycin and intermediately resistant to kanamycin [25].

The identification of beneficial mutations and screening of high PHB-yield microorganisms are important for the successful development of various strains in the fermentation industry. To improve the production of PHB, the highest PHB producing *A. haemolyticus* (HO1) strain was exposed to UV irradiations at 11.5 distance for 5 and 10 min ranges. Mutants were isolated and were compared with wild type for total yield of PHB. Results indicated that survival rate for *A. haemolyticus* (HO1) was 0.03% on 5 min UV exposure, while 10 min UV exposure showed the survival rate of 0.013%.

The amount of PHB extracted from *A. haemolyticus* (HO1) strain before and after UV irradiation were increased substantially from 50% to 67% in comparison to wild type. Increase in two fold PHB production has been reported earlier [26, 27, & 28].

Total cell protein profile analysis (SDS-PAGE) of *A. haemolyticus* (HO1) parental and mutants (three mutants resulted from five min. (M 6.5, M 3.5 and M 2.5), and from 10 min (M 2.10, M 3.10 and M 36.10) strains was carried out to determine distances/similarities. The results showed that protein profile of (M 3.10) mutant was closely resembling that of parental strain, where the percentage of difference was 44% and that of similarity was 56%. Our results are in accordance with the published study of [29] where they showed the mutant strain protein profiles to closely resemble those of parental strain.

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