

## Isolation and Identification of Polyhydroxybutyrate (PHB)-Producing Microorganisms from Various Sources in North Sinai

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### ABSTRACT

The amount of non-biodegradable plastic waste on our planet is enormous. Natural materials like biomaterials are among the finest replacements for manmade plastics. In order to assist microorganisms resist severe environments, certain microbes generate bioplastics, which are lipid polyesters that build up as storage materials. The primary goal of this investigation was to isolate and characterize cultivable bacteria and fungi capable of producing bioplastics from North Sinai from different sources, such as saline soil, olive pomace, landfills, and seawater. Seven bacterial and five fungal isolates were selected from a total of 108 isolates to assay for PHB production and the selected isolates were stained with Sudan Black B for PHB formation, while Nile Blue A staining was used to detect the presence of PHA granules. All promising bacterial isolates with the highest PHB accumulation were identified as *Halomonas*, *Lysinibacillus*, *Mesobacillus*, *Paracoccus*, *Paraliobacillus*, *Glutamicibacter*, and *Aquamicrobium*; most fungal isolates were yeasts, identified as *Rhodotorula*, *Hortaea*, *Meyerozyma*, and *Sarocladium* by morphological and biochemical characterization and confirmed by molecular techniques.

**Keywords:** Bacteria; Fungi, North Sinai; Polyhydroxybutyrate (PHB).

### INTRODUCTION

One of the most serious environmental challenges confronting humanity today is the persistence of man-made plastics in the environment (Mannina *et al.*, 2019). Plastics are everyday objects; they are present in almost every aspect of modern life and are used in all countries (Clunies, 2019; Asiandu, 2021). Synthetic plastic waste often harms the air, land, and water ecosystems (Asiandu *et al.*, 2021). Non-biodegradable polymer accumulation in landfills is dangerous to the environment as well as human health (Khomlaem *et al.*, 2022). Plastics Europe (2019) reported that global plastics manufacturing reached 348 million tonnes in 2017, which was a 4% increment compared to the previous year. But Asiandu *et al.* (2021) predicted that the number of plastics made will rise over the next 20 years. There has been a lot of interest in microbially produced biopolymers in recent years because of their great sustainability and minimal toxicity, which can aid in reducing environmental waste disposal (Khomlaem *et al.*, 2022). More than 300 species, including bacteria (both Gram-negative and Gram-positive) (Anjum *et al.*, 2016) and archaea (Hermann-Krauss *et al.*, 2013), have been identified to accumulate PHAs (Zinn *et al.*, 2001) under both aerobic and anaerobic conditions (Kim *et al.*, 2007). The ability of bacteria, yeast, fungi, and archaea to polyhydroxybutyrate formation and polyhydroxyalkanoates accumulation under hard conditions when other nutrients are lacking and the carbon supply is sufficient is the fundamental route for microorganism survival (Luengo *et al.*, 2013; Thapa *et al.*, 2018; Mostafa *et al.*, 2020). Different bacteria produce different PHAs as Poly[R-3-hydroxybutyrate] (PHB) is the most frequent and the first form of PHA identified. It is more environmentally friendly than petroleum (Luengo *et al.*, 2013; Thapa *et al.*, 2018). PHB generated by microbes could be used as a plastic raw material (Yanti *et al.*, 2019). PHB is a thermoplastic polyester with biodegradable and biocompatible features as well as physical attributes similar to polypropylene (Yanti *et al.*, 2021). Because of their rapid environmental decomposition, PHBs have been considered a substitute for the production of biodegradable

polymers (Danial *et al.*, 2021). In aerobic conditions, PHB degraded totally into CO<sub>2</sub> and water (Hankermeyer and Tjeerdema, 1999). The primary goals of this study were to isolate and characterize a variety of PHB-producing microorganisms from distinct geographical sites in North Sinai.

### MATERIALS AND METHODS

#### Sample Collection

Samples of saline soil, olive pomace, landfill sites, and seawater were collected in sterile plastic bags from and around Arish city, North Sinai governorate at January 2021 (Fig.1). Samples were transferred into sterile plastic bags and was Kept till use, at 4 °C.

#### Isolation technique

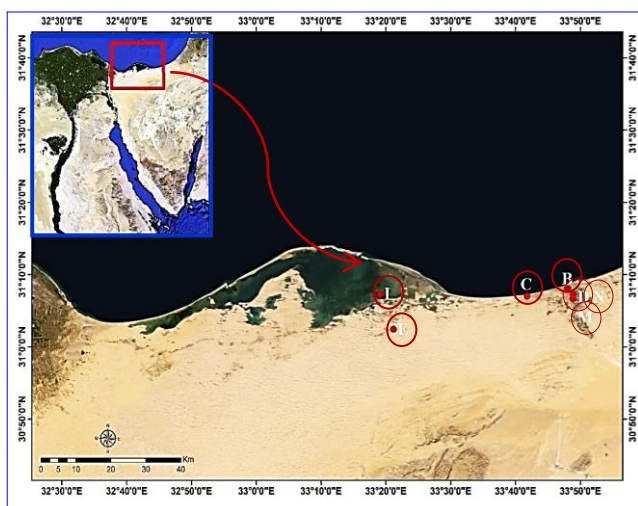
The samples were prepared sequentially by vigorously mixing 10 g of each sample in 100 ml of sterile sea water , and then 0.1 ml was streak-plated onto Nutrient Agar (N.A.) medium. The medium was supplemented with 4% NaCl for salt and seawater samples. The plates were then incubated for 48 hours at 37 °C. Colonies with distinguishing characteristics were chosen, purified, and kept at 4 °C on Nutrient agar slants. For fungal isolation, streak plate method was performed with 0.5 ml of each sample on Potato Dextrose Agar (PDA) medium supplemented with 4% NaCl for saline samples, then the plates were incubated at 25 °C for 4–7 days. Pure cultures were separated and kept at 4 °C.

#### Screening for PHB -producing microorganisms

##### Bacterial isolates

A total of 50 bacterial isolates were chosen based on morphological characteristics and cultured for 24hr at 37 °C on N.A. medium enriched with 1% glucose as a source of carbon (Luhana and Patel, 2013). Sudan Black B (0.05%) was applied for 30 minutes to all culture plates before being washed with ethanol. Positive results were represented by plates that were black or dark blue (Luhana and Patel, 2013; Mostafa *et al.*, 2020a; 2020b). For best confirmation, the isolates that showed a positive result with Sudan Black B stain were re-stained with alcoholic Nile Blue A stain (1%), and the plates were subjected to

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**Figure (1):** Location map of sampling sites in the North Sinai, Egypt. I, North Sinai traveling road (Tofaha city); B, waste dumping place; C, seawater, E, solar saltern; D, olive oil pomace; M, olive oil pomace (Jan. 2019) and N, olive oil pomace (Mar. 2018).

365 wavelengths of ultraviolet light. The plates with blue fluorescent showed a positive result (Sohail *et al.*, 2020).

#### *fungal isolates*

To detect bioplastics accumulation in fungi, 58 fungal isolates were tested for PHB synthesis using the biofuel screening method (Kamoun *et al.*, 2018). At first, a few microliters of Sudan Black B stain (0.3 g in 100 ml 70% ethanol) were applied to a slide and dried for 20 minutes at 50 °C. The excess stain on the slide was removed with 70% ethanol, and then for a few seconds, the slides were counterstained with safranin (0.5 g in 100 ml distilled water). The slides were cleaned with distilled water, dried, and examined under a light microscope (Kamoun *et al.*, 2018). Sudan Black B staining revealed positive results on fungal isolates. Nile Blue A staining was performed on each positive PHB accumulating fungi as a conformation test. Heat-fixed smears of the isolates were flooded with 1 % Nile Blue A stain, heated for 10 min, and the slides were washed with 70% ethanol to remove the excess stain. Then the slides were examined under the fluorescence microscope using a green filter (365 wavelengths). Each stained slide with Nile Blue A was examined under a fluorescent microscope (Carl ZEISS). Cells which were exhibited orange or yellow fluorescence were considered positive for PHB accumulation (Rawte and Mavincurve, 2002).

#### **Preliminary identification of potential isolates-PHB producer**

Gram stain, motility, indole, ornithine, urease, oxidase, catalase, amylase, carbohydrate catabolism tests, and citrate hydrolysis were used to identify promising isolates (Akel *et al.*, 2008; Leboffe and Pierce, 2011).

#### **Molecular identification of selected PHB-producing isolates**

##### *DNA extraction*

Genomic DNA was extracted from bacterial and fungal strains during their exponential development phase. with the recommendations provided by the manufacturer, the ABT DNA micro-extraction kit (Applied Biotechnology Co. Ltd., Egypt). 16S rRNA and 18S rRNA gene

sequences for bacterial and fungal molecular identification, respectively, were used. The 16S rRNA gene was isolated using a set of universal primers (Invitrogen, USA): bacterial universal primers, forward: 27F (5-AGA GTT TGA TCC TGG CTC AG-3), reverse: 1492R (5-GGT TAC CTT GTT ACG ACT T-3), and 18S rRNA for fungal primers, forward: ITS1 (5-TCC GTA GGT GAA CCT GCG G-3), reverse: (5-TCC TCC GCT TAT TGA TAT GC-3). For the PCR, the following conditions were employed: 30 cycles of 95°C pre-denaturation for 5 minutes, 94°C denaturation for 1 minute, 60°C annealing for 1 minute, 72°C extension for 1 minute 30 seconds, 72°C final extension for 10 minutes, 4°C final extension for 10 minutes in a 50-l reaction system. The samples were made in accordance with the Macro Gen Company's specifications, with 50 ng/l of each PCR product. The evaluation of the sequences was performed using BLAST (<http://www.ncbi.nlm.gov/BLAST>).

##### *Phylogenetic construction*

The bacterial and fungal sequences used for phylogenetic analysis alignment by clustal W and used methods such as neighbor-joining to identify the evolutionary tree by MEGA.X software (Tamura *et al.*, 2013; Ali *et al.*, 2018; Elshafey *et al.*, 2022).

##### *Accession numbers for nucleotide sequences*

All the data from this study with 16S rRNA and 18S rRNA gene sequences were uploaded to the NCBI and GenBank nucleotide sequence databases.

## **RESULTS**

### **Isolation and selection of bacterial isolates capable of producing PHB**

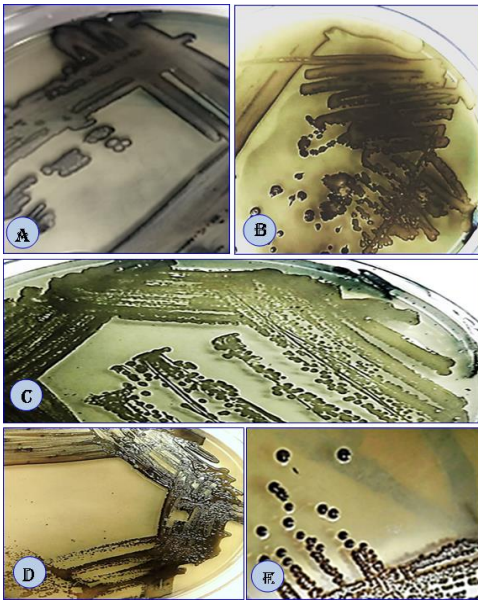
Out of 50 bacterial isolates collected and screened for their capacity to accumulate polyhydroxybutyrate (PHB). Only seven bacterial isolates (C1, D2, E1, E3, E5, I3, and M2) displayed the potential to accumulate PHB using Sudan Black B stain (Fig. 2) and were validated using Nile Blue A stain.

### **Isolation and selection of fungal isolates capable of producing PHB**

Five of fungal isolates out of 58 total fungal isolates demonstrated the ability to accumulate PHB. The positive isolates (B1, B11, E1, D6, and N8) were stained with Sudan Black B (Fig. 3), and all five isolates were retested with Nile Blue A. The isolates (B3, B11, and N8) showed a clear positive result for PHB accumulation with bright yellow fluorescence, whereas (E1 and D6) showed a low amount of fluorescence compared to the other strains (Fig. 4).

### **Identification of promising isolates that produce PHB**

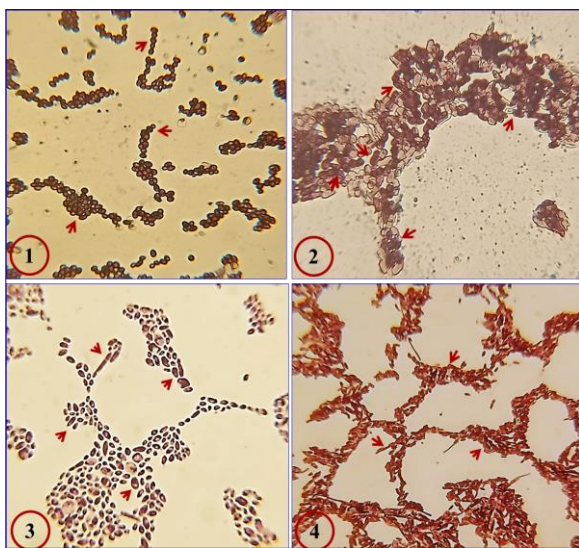
Morphological characterizations of the selected bacterial isolates are bacilloid form with varied ability to Gram stain where four bacterial isolates are Gram-positive (D2, E1, E5 and I3), while the rest of are Gram-negative. Biochemical characterizations of these isolates are listed in Table (1). Meanwhile, all selected fungal isolates showed single cell with typical morphological character of yeast. Biochemical characterization and ability for sugar utilization are listed in Table (2).



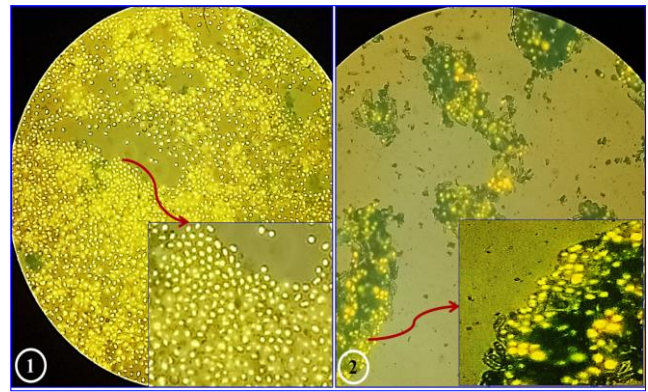
**Figure (2):** Bacterial isolates stained with Sudan Black. Colonies that stained black or dark blue indicated the ability of PHB-production. Bacterial isolates designated as follow: A, C1; B, D2; C, E1; D, E3; and E, M2.

### Molecular identification of the promising bacterial and fungal-PHB producers

Molecular characterization of PHB-producing isolated revealed that bacterial isolates are belonging to *Halomonas*, *Lysinibacillus*, *Mesobacillus*, *Paracoccus*, *Paraliobacillus*, *Glutamicibacter*, and *Aquamicrobium*, for isolate C1, D2, E1, E3, I3 and M2, respectively. Meanwhile, for isolated yeast that showed a high degree of similarity (>95% similarity) are belonging to the following genera: *Rhodotorula*, *Hortaea* and *Meyerozyma* for isolate B3, B11, B6 and E1, respectively, and *Sarocladium* for fungal isolate N8. The phylogenetic relationships of the isolated strains are represented in (Fig. 5 and 6), and sequences with accession numbers are in (Table 3).



**Figure (3):** Selected PHB-producing fungi stained with Sudan Black B and viewed using a light microscope at magnification 40X. 1, B3; 2, B11; 3, E1 and 4, N8.



**Figure (4):** Fungal isolates stained with Nile Blue A and viewed using fluorescence microscope at magnification 40 X. Arrows referred to magnified fungal cells. 1, B3 and 2, B11.

### DISCUSSION

Microorganisms respond to environmental stress by producing and accumulating PHB, which helps them survive in challenging environments (Juengert *et al.*, 2018). Furthermore, due to the harsh environmental circumstances, particular genes and enzymes in microbes may be able to produce significant amounts of PHB. Because of the enormous diversity of microorganisms, it is necessary to constantly identify and screen for those that can produce significant amounts of PHB from low-cost sources of nutrients. As a result, isolates capable of producing polyester were found in North Sinai's saline soil, olive pomace, landfills, and seawater (Alzubaidy *et al.*, 2016). These results agree with the results of (Luengo *et al.*, 2013; Thapa *et al.*, 2018; Mostafa *et al.*, 2020a) which demonstrated that PHB-producing bacteria thrive in ecosystems with stress and poor dietary requirements.

Moreover, Bacteria that succeeded in such ecosystems should have adapted their metabolisms to variations in salt concentrations, excess carbon sources, and limited amounts of other essential nutrients such as nitrogen or phosphorous. Several halophilic and halotolerant bacteria can tolerate a wide range of NaCl concentrations (Oren 2008), whereas various species accumulate polyesters (Quillaguamán *et al.*, 2010). In this regard, 14% of the bacterial strains showed their ability to accumulate PHB, namely *Paracoccus onubensis* family: Rhodobacteraceae, *Aquamicrobium defluvii* family: Phyllobacteriaceae, and *Halomonas venusta* family: Halomonadaceae were Gram-negative bacteria, while *Mesobacillus jeotgali*, *Lysinibacillus fusiformis*, and *Paraliobacillus quinghaiensis* were all Gram-negative bacteria belonged to the family: Bacillaceae, while *Glutamicibacter arilaitensis* family: Micrococcaceae were Gram-positive bacteria. On the other hand, 8.6% of the fungal isolates originated from fungal PHB-producing organisms, namely: *Rhodotorula mucilaginosa* family: Sporidiobolaceae, *Hortaea werneckii* family: Teratosphaeriaceae, *Meyerozyma caribbica* family: Saccharomycetaceae, *Sarocladium strictum* order: Hypocreales, and *Meyerozyma carpophila* family: Debaryomycetaceae.

**Table (1):** Morphological and biochemical characterization of PHB-Producing bacterial isolates.

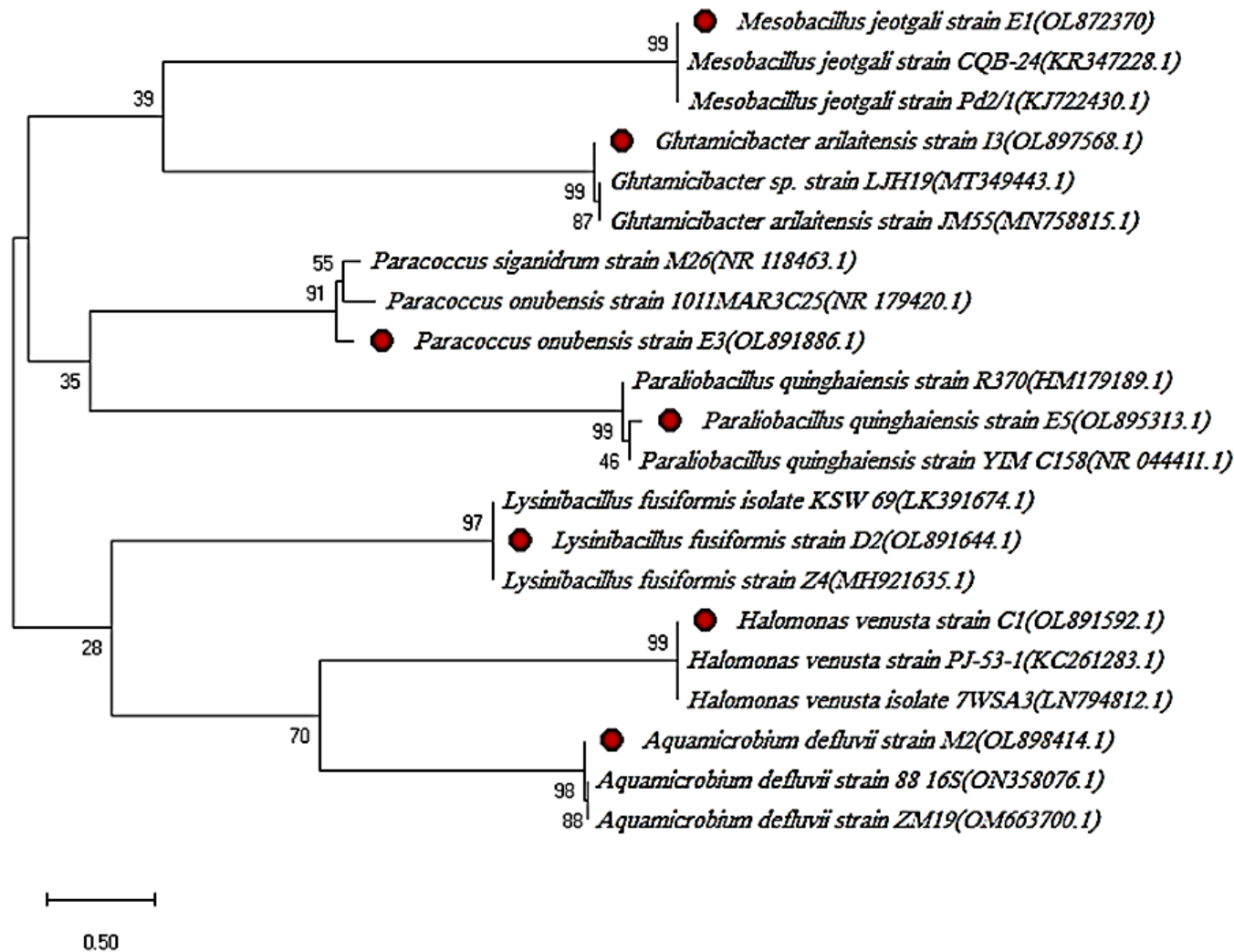
Tested characters	PHB-Producing bacteria <sup>†</sup>						
	C1	D2	E1	E3	E5	I3	M2
<b>Gram stain</b>	-	+	+	-	+	+	-
<b>Shape</b>	Rod	Rod	Rod	Short Rod	Rod	Coccobacilli	Rod
<b>Endospore formation</b>	-	+	+	-	+	-	-
<b>Motility</b>	+	-	+	-	+	-	+
<b>Catalase production</b>	+	+	+	+	+	+	+
<b>Oxidase production</b>	+	+	+	+	-	-	+
<b>Ornithine production</b>	+	+	+	+	+	+	+
<b>Urease</b>	+	+	-	+	+	-	-
<b>Indole production</b>	-	-	-	-	-	-	-
<b>Carbohydrate utilization</b>							
<b>Glucose</b>	-	+	+	+	+	+	+
<b>Dextrose</b>	-	-	+	+	-	+	+
<b>Lactose</b>	-	+	-	+	-	+	-
<b>Maltose</b>	-	+	+	+	+	-	-
<b>Cellobiose</b>	+	+	+	-	+	-	-
<b>Starch</b>	-	-	+	-	-	+	+

<sup>†</sup>+, Positive ability; -, negative.

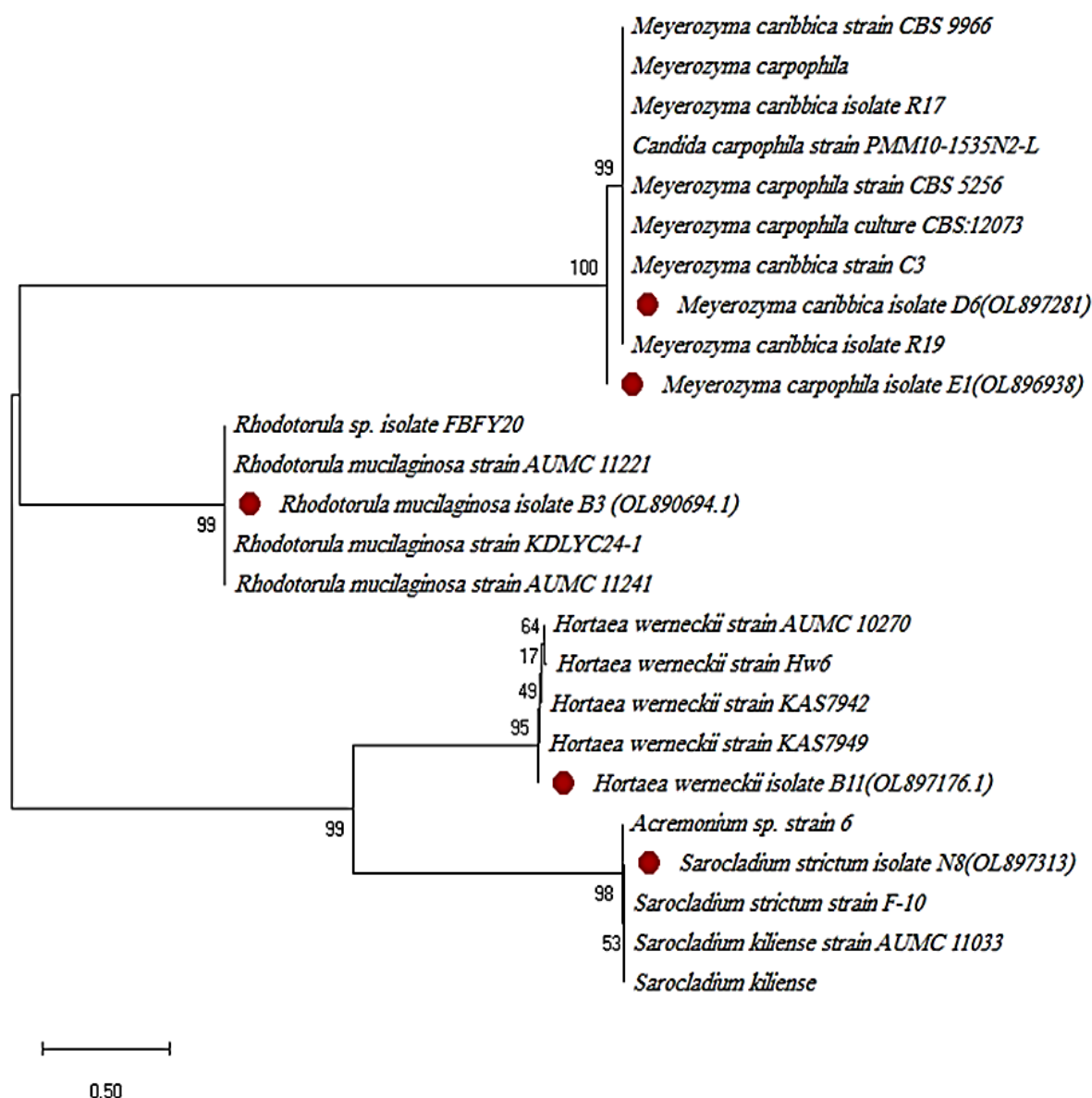
**Table (2):** Morphological and biochemical characterization of PHB-Producing fungal isolates.

Tested characters	PHB-Producing fungi				
	B3	B11	D6	E1	N8
<b>Colony color</b>	Orange	Black	White	White	White
<b>Catalase</b>	+	+	+	+	+
<b>Protease</b>	-	-	+	+	+
<b>Urease</b>	+	+	-	-	+
<b>Indole</b>	-	-	-	-	-
<b>Ornithine production</b>	+	+	+	+	-
<b>Citrate utilization</b>	-	-	+	-	+
<b>Carbohydrate utilization</b>					
<b>Glucose</b>	+	+	+	+	+
<b>Dextrose</b>	+	+	G+	G+	+
<b>Lactose</b>	-	+	-	-	-
<b>Maltose</b>	-	-	-	-	-
<b>Cellobiose</b>	-	+	+	+	+
<b>Starch</b>	-	-	-	-	+

-, Negative; G, Gas production



**Figure (5):** Phylogenetic study of PHB-producing bacterial isolates based on the 16S rRNA nucleotide sequence. The scaled bar represented 0.10 nucleotide substitutions per location in the sequence. The numbers on the nodes represent the percentage of bootstrap support based on neighbor-joining analysis of 1000 resampled datasets.



**Figure (7):** Phylogenetic study of fungal isolates based on the 18S rRNA nucleotide sequence. The scaled bar represented 0.10 nucleotide substitutions per location in the sequence. The numbers on the nodes represent the percentage of bootstrap support based on neighbor-joining analysis of 1000 resampled datasets.

On the other hand, the current study's findings suggested that *Halomonas venusta* had the highest concentration of PHB-production and were consistent with (Gao and Zhang, 2014). Besides this finding in agreement with (Elshafey et al., 2022), the *Halomonas* strain was identified as halophilic bacteria with valuable biotechnological activity. On the other hand, the genus *Lysinibacillus* has been shown to accumulate PHB polymer (Lathwal et al., 2018). Furthermore, our research discovered that *Paracoccus onubensis* (strain E3) had a 99.24% resemblance to a newly isolated species described by (Gutierrez et al., 2002). However, the orange yeast *Rhodotorula mucilaginosa* was mentioned as a PHB-accumulating yeast by (Nantha et al., 2018). Furthermore, the black yeast *Hortaea werneckii*

induced the superficial mycosis *Tinea nigra*. This asymptomatic condition often affects human palms and soles (Bonifaz et al., 2008). While in the current study's findings suggested that it could be useful yeast in the future as a PHB producer.

On the other hand, olive pomace is a frequent waste product from agriculture in the Mediterranean region, as well as a byproduct of olive oil production. Depending on how it is extracted, olive oil can include a substantial volume of oil as well as a significant amount of moisture (Maragkaki et al., 2016). The majority of freshly generated olive pomace in Egypt is piled up near olive mills. The North Sinai governorate is distinguished by olive oil farming and extraction, according to (Hanene et al., 2015). Olive oil agriculture is an important component of Mediterranean land use.

Table (3): Molecular identification, based on 16S rDNA sequencing data, of the isolated PHB-microbial producers.

Isolates	Identified name	Similarity %	NCBI Accession number
<b>Bacteria</b>			
C1	<i>Halomonas venusta</i>	99.86%	OL891592
D2	<i>Lysinibacillus fusiformis</i>	99.93%	OL891644
E1	<i>Mesobacillus jeotgali</i>	99.65%	OL872370
E3	<i>Paracoccus onubensis</i>	99.24%	OL891886
E5	<i>Paraliobacillus quinghaiensis</i>	99.60%	OL895313
I3	<i>Glutamicibacter arilaitensis</i>	99.86%	OL897568
M2	<i>Aquamicrobium defluvii</i>	99.39%	OL898414
<b>Yeast</b>			
B3	<i>Rhodotorula mucilaginosa</i>	99.67%	OL890694
B11	<i>Hortaea werneckii</i>	98.73%	OL897176
D6	<i>Meyerozyma caribbica (Candida fermentati)</i>	99.64%	OL897281
E1	<i>Meyerozyma carophila</i>	99.63%	OL896938
<b>Mold</b>			
N8	<i>Sarocladium strictum</i>	99.63%	OL897313

Similarly, *Sarocladium strictum* and *Aquamicrobium defluvii* were isolated from olive pomace and demonstrated the ability to manufacture and accumulate PHB under standard growth conditions (Kimura *et al.*, 2004).

## CONCLUSION

The use of nondegradable plastics, which are currently used in many industries purpose has become a serious global problem, due to its accumulation in the environment. Bioplastics, also known as biodegradable PHBs of microbial origin, are thought to offer a feasible replacement. Therefore, the goal of the current investigation was to investigate and characterize the most efficient polyhydroxybutyrate accumulating bacterial and fungal strains from various sources in North Sinai. The study revealed that the most potent PHB-bacterial isolates are belong to the following genera: *Halomonas*, *Lysinibacillus*, *Mesobacillus*, *Paracoccus*, *Paraliobacillus*, *Glutam-icibacter*, *Aquamicrobium*. The study also revealed the ability of some yeast to produce PHB comparable to the identified bacteria isolates. These yeast isoates are belonging to: *Rhodotorula*, *Hortaea*, *Meyerozyma*, and *Sarocladium* species.

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## عزل وتوصيف الكائنات الحية الدقيقة المنتجة للبلاستيك الحيوي من أماكن متنوعه بشمال سيناء

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### الملخص العربي

يتراكم على كوكب الأرض كمية هائلة من النفايات البلاستيكية الغير قابلة للتحلل. وتعتبر المواد الحيوية، وهي مواد طبيعية، من أفضل البدائل للبلاستيك الصناعي. ولذلك تنتج بعض الميكروبات مواد بلاستيكية حيوية، وهي بوليستر دهني يتراكم كمواد تخزين داخل الخلايا لمساعدة الكائنات الحية الدقيقة على تحمل البيئات القاسية. لذلك كان الهدف الأساسي من هذه الدراسة هو عزل وتوصيف البكتيريا والفطريات والخمائر القادرة على إنتاج البلاستيك الحيوي من مصادر مختلفة بشمال سيناء، مثل التربة المالحة وثقل الزيتون ومكبات النفايات ومياه البحر. وتم اختيار سبع عزلات بكتيرية وخمسة عزلات فطرية من إجمالي 108 عزلة لفحص إنتاج PHB وتلون العزلات المختارة بصبغة Sudan black B لتكوين PHB، بينما تم استخدام صبغة النيل الأزرق A لتأكيد تكوين وتواجد حبيبات PHA. وتم التعرف على جميع العزلات البكتيرية الواعدة ذات تراكم أعلى من PHB مثل *Halomonas*، *Lysinibacillus*، *Mesobacillus*، *Paracoccus*، *Paraliobacillus*، *Glutamicibacter*، *Aquamicrobium* وكانت معظم العزلات الفطرية عبارة عن اربعة من الخمائر تم تحديدها على أنها *Rhodotorula* و *Hortaea* و *Meyerozyma* بالإضافة لفطر *Sarocladium* عن طريق التوصيف المورفولوجي والكيميائي الحيوي وتم تأكيدها بالتقنيات الجزيئية.