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Isolation of Rhizobia from Species of Legumes Plants and Utilizing them in Ploy-β-Hydroxybutyrate Production (PHB)

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عزل بكتيريا العقد الجذرية من أنواع من النباتات البقولية واستعمالها في انتاج متعدد هيدروكسي البيوترات *Poly- β-Hydroxybutyrate (PHB)*

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Abstract:

Thirty-eight rhizobia isolates were obtained from legume species included: *Medicago sativa*, *M. littoralis*, *Vicia faba*, *Lupinus varius*, *Cicer arietinum*, *Arachis hypogaea*, *Vigna unguiculata* and *Pisum sativum*. They were collected from different regions of Libya with arid and semi-arid climate. The All-Rhizobia isolates were able to form effective symbiosis with their hosts. Isolates grown on yeast extract and mannitol medium were primary screened for their ability to produce polyhydroxybutyrate on the basis of staining with Sudan black B. Different sources of carbon and nitrogen were added to the yeast extract and mannitol medium. The growth of isolates varied in using these media where the Yeast extract and mannitol supplemented with 2.5 g of Tryptone and Peptone was the best medium to support the growth of the isolates. Temperature and pH have not had a significant effect on the ability of isolates to produce polyhydroxybutyrate. The moderate and slow growing isolates, especially RVF1b, RC1b, RC3 and RVG5 were superior in the producing of the polyhydroxybutyrate under all conditions compared to the other isolates, while the isolate RP4 was the least productive. The producing polyhydroxybutyrate in this study have not shown any toxic effect on human blood, as no clotting has occurred after one hour of incubation.

Keywords: Rhizobia isolate, Fezzan, Legumes, Sudan black stain, Polyhydroxybutyrate.

الملخص

ثمانية وثلاثون عزلة ريزوبية تم الحصول عليها من أنواع من البقوليات: *Medicago sativa*، *M. littoralis*، *Vicia faba*، *Lupinus varius*، *Cicer arietinum*، *Arachis hypogaea*، *Vigna unguiculata* و*Pisum sativum*. جمعت من مناطق مختلفة من ليبيا ذات مناخ جاف وشبه جاف. جميع العزلات استطاعت أن تكون تكافل فعال مع عوائلها،

غربلت العزلات النامية الوسط الزرعي مستخلص الخميرة والمانيتول غربلة اولية على اساس الاصطباغ بصبغة Sudan black B وقدرتها على انتاج متعدد هيدروكسي البيوترات. اضيف للوسط مصادر مختلفة للكربون والنيتروجين، فكانت العزلات متباينة في استخدام هذه الاوساط. درجة الحرارة وألاس الهيدروجيني لم بكون لها تأثير يذكر على قدرة العزلات على انتاج المتعدد. العزلات المتوسطة والبطيئة النمو، خاصة RVF1b، RC1b، RC3 وRVG5 كانت متميزة في انتاج ذاك المتعدد تحت جميع الظروف بالمقارنة مع العزلات الأخرى، بينما كانت العزلة RP4 الاقل إنتاجاً. وسط مستخلص الخميرة والمانيتول المضاف إليه 2.5 جرام من Tryptone وPeptone كان الأفضل مع جميع العزلات. لم يظهر المتعدد أي تأثير سام في دم الأنسان، فلم يظهر تخثر بعد ساعة من التحضين.

الكلمات المفتاحية: عزلة ريزوبية، فزان، بقوليات، صبغة اسود سودان، متعدد هيدروكسي البيوترات.

Introduction

Plastic materials have become an integral part of our daily lives that are used in various industrial fields. Despite the common use of the plastics, they are considered one of the most important environmental problems that threatening human health; because plastics do not decompose in nature (Babu et al., 2014, Salem, & Salem., 2025). Due to the many problems of plastics, the recent studies began to search for alternatives, and they found an alternative in biopolymers that produced by microorganisms, which are naturally decomposable into water and carbon dioxide (Babu et al., 2014).

Biopolymers are produced by the growth of microorganisms on natural materials as the microbes produce different types of these polymers, such as Polyhydroxyalkanoate (PHA), which accumulate in cells as a source of energy and carbon, and their accumulation increases when there is a deficiency of nutrients in the growth medium. Most of the PHAs produced by microorganisms are polyhydroxybutyrate (PHB) which is a short-chain type and may reach for 90% of the dry cell weight in some types of bacteria (Aswini et al., 2014; Wang & Yu, 2000; Wang et al., 2007). The polyhydroxybutyrate is a source of energy and a regulator of oxidation within the cell and its absence in damaged living cells leads to the degradation of other cellular components such as RNA and protein[6]. The polyhydroxybutyrate in microbes plays a role similar to the role of fats in the human body and starch in plants (Chandrashekharaiah, 2007; Lee, 1996; Macario, 2009).

The biosynthesis of Polyhydroxybutyrate inside the cell depends on the activity of enzymes, as there are three enzymes are responsible for the production of Polyhydroxybutyrate which are including: β-ketoacyl-CoA thiolase, acetoacetyl-CoA reductase and PHB polymerase. These enzymes work sequentially, starting with the enzyme of β-ketoacyl-CoA thiolase, which is responsible for producing acyl-CoA and acetyl-CoA and then it links two acetyl-CoA molecules with a carbon bond to produce acetoacetyl-CoA. The second step is catalyzed by the enzyme of acetoacetyl-CoA reductase, which works to reduce acetoacetyl-CoA to 3-hydroxybutyryl-CoA in the presence of the coenzyme NADPH2+ (Tetsuya et al., 1987). The final step relies on the enzyme of PHB polymerase which polymerizes the single units of 3-hydroxybutyryl after removal of CoA to produce PHB polymer (Wang, 2008).

PHB-producing microorganisms include more than 90 species, comprising aerobic and anaerobic bacteria, archaea and photosynthetic bacteria (Chandrashekharaiah, 2007; Kocharin, 2013). The bacterial species that produce PHB polymer include: *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Micrococcus* and *Rhizobium* (Charen et al., 2014).

Rhizobia are Gram-negative, motile, non-spore-forming rod-shaped bacteria that resident in soil and grow in the laboratory on yeast extract and mannitol medium. Rhizobia use many carbohydrates as a carbon source and often produce acid but do not form gas (Alexander, 1979; Quan et al., 2005). Rhizobia can grow on the nodules of some plant roots to fix the nitrogen or freely in the soil as decomposer bacteria (Trinick, 1982; Young, 1992).

Because of the environmental problems caused by synthetic plastics, the increasing global demand for biopolymers and the lack of studies on this subject in our region, the aim of this

study was to obtain rhizobia isolates from different types of leguminous plants that may be able to produce Polyhydroxybutyrate, in addition to study the best conditions for the producing of this Polyhydroxybutyrate and testing its toxicity.

Methods

Nodule's collection

Thirty-eight root nodules belonging to different types of leguminous plants were obtained from the research laboratory of the Department of Botany - Faculty of Science - University of Sebha, collected from different regions of Libya with arid and semi-arid climate, including Jabal al-Hasawna, Taroot Ishati, Sebha and Tesawa in Wadi Etba of the southwest of Libya, and Wadi Barsas in the far northeast of Benghazi (Fig. 1).

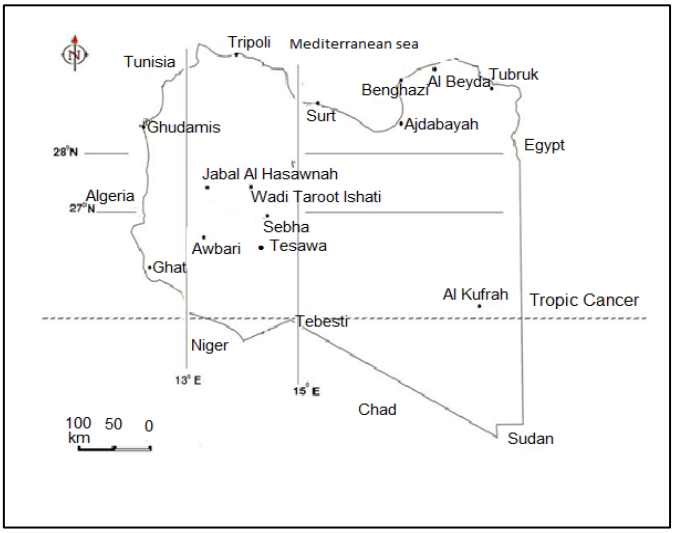

Fig. 1. Map of Libya showing the locations of the root nodules collection.

Isolation and Authentication of Root-nodule Bacteria

Yeast Extract Mannitol Agar (YEMA) was used to isolate and preserve rhizobia. YEMA contains the following compounds (g/L): Mannitol (10), Yeast extract (1), NaCl (0.1), MgSO4. 7H2O (0.2), K2HPO. 3H2O (0.46), K2HPO (0.12), pH 7.2 (Charen et al., 2014). Bacteria were isolated from root nodules and the isolates were tested on their original hosts by applying Koch's postulates; to confirm that they are rhizobia and verify their ability to form symbiosis (Hungria et al., 2016; Vincent, 1970).

The utilizing medium for the production of polyPHB

In the primary screening for the production of polyPHB, YEMA medium was used, in addition to that, four other modified media were used (Mercan et al., 2002), in the second medium Tryptone (2.5g), Peptone (2.5g) were added. The third medium was supplemented with Tryptophan (10g) and the mannitol was replaced by Glucose (10g), while in the fourth medium mannitol was replaced by Sucrose (10g) and L-Glutamine (10g) was added. The fifth medium mannitol sugar was replaced by Sucrose (10g), in addition to adding of the Proline (10g).

Test of PHB formation in bacterial cells

Two methods were used to verify the ability of isolates to produce Polyhydroxybutyrate. Firstly, The Petri dish immersion method was applied as described by Liu et al. (1998). The glass slide staining method with Sudan Black B (Kumari & Dhingra, 2013) and Nile blue (Ostle & Holt, 1982) were used as well.

Studying of the optimal condition for PHB production

Optimum temperature

The second medium was used to culture the isolates and then they were incubated at 37°C and 40°C for 48 hours. The formation of PHB was examined by carrying out the Petri dish staining method (Nair et al., 1993).

Optimal pH

To verify the Optimal pH for the production of PHB the second medium was utilized after adjusting its pH at pH 5 and pH 9 and then the isolates were incubated at 30°C for 48 hours, then the formation of PHB was tested by Petri dish staining method (Nair et al., 1993).

PHB extraction by Sodium hypochlorite–Chloroform method

The extraction of PHB was performed by inoculating 100 ml of the fourth medium in a 250 ml flask with rhizobial isolates at 1% inoculum (Hahn et al., 1993; Soam et al., 2012).

PHB toxicity test

The toxicity of PHB was examined by mixing 5 ml of human blood with 10 ml of citric acid–dextrose solution, and they were dissolved in 75 ml of distilled water (Qu et al., 2006). The toxicity was checked depends on the changes in the appearance of the blood with the presence or absence of coagulation.

Results and Discussion

Research on rhizobia in Libya is very limited as it does not exceed 1% of the total leguminous plants, which are their number is more than 208 species in the country. On the of that the studies that focus on the manufacture of the Polyhydroxybutyrate by rhizobia are minor compared to the research that involved in the production of Polyhydroxybutyrate by the other rod-shaped bacteria as *Bacillus* and *Escherichia*; therefore, this investigation could be considered a first step in our region to shield the light on the productions of rhizobia isolates (Al-Idrissi et al., 1996).

Cultural characteristics of colonies and germination test

The isolates showed rhizobia characteristics, for instance gum production and straight edge with different colors (Fig. 2), in addition to the time of colony emergence and nodule formation on the plant. Some isolates were *large in size with a diameter (>1 mm* after 48 h), while others were small. Some of the isolate's characteristics are recorded in (Table 1), which also shows the optimum conditions for PHB production that displayed by the isolates.

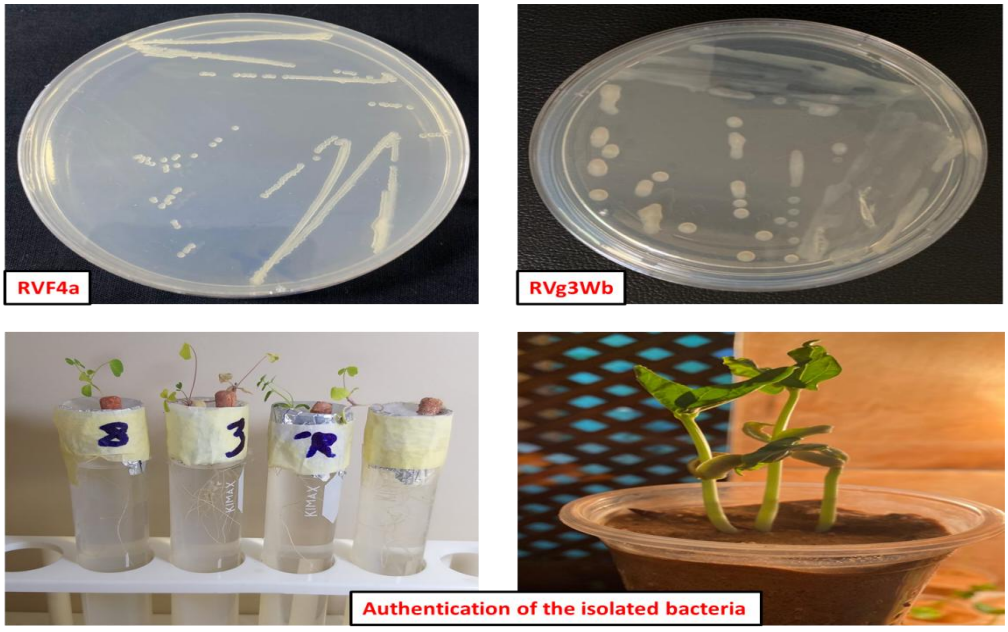

Fig. 2. Colony morphology on YEM medium, with germination test.

Table 1. Host Plant, Site of isolation and colonial features of isolates.

Isolates	Host Plant	Site of isolation	Colonial features			
			color	size	Gram reaction	Growth period (days)
R1	***Medicago sativa***	**Taroot Ishati**	white	big	-ve	1-3
R2	***M. sativa***	**Taroot Ishati**	white	big	-	1-3
R3	***M. sativa***	**Taroot Ishati**	white	big	-	1-3
R4a	***M. sativa***	**Taroot Ishati**	white	big	-	1-3
R4b	***M. sativa***	**Taroot Ishati**	whitish yellow	big	-	1-3
R5	***M. sativa***	**Sebha**	white	big	-	1-3
R7	***M. sativa***	**Sebha**	white	big	-	1-3
R8	***M. sativa***	**Sebha**	white	big	-	1-3
R9	***M. littoralis***	**Barsas**	white	big	-	1-3
R10	***M. littoralis***	**Barsas**	white	big	-	1-3
R11	***M. littoralis***	**Barsas**	white	big	-	1-3
R12	***M. littoralis***	**Barsas**	white	big	-	1-3
R13	***M. littoralis***	**Barsas**	white	big	-	1-3
R14	***M. littoralis***	**Barsas**	white	big	-	1-3
RVF1a	***Vicia faba***	**Tesawa**	white	small	-	1-3
RVF1b	***V. faba***	**Tesawa**	translucent	big	-	1-3
RVF2	***V. faba***	**Tesawa**	translucent	moderate	-	1-3
RVF4a	***V. faba***	**Sebha**	white	small	-	1-3
RVF4b	***V. faba***	**Sebha**	brown	small	-	1-3
RVF5	***V. faba***	**Tesawa**	creamy	small	-	1-3
RL	***Lupinus varius***	**Jabal al-Hasawna**	whitish brown	moderate	-	1-3
RC1a	***Cicer arietinum***	**Tesawa**	creamy	small	-	4-6
RC1b	***C. arietinum***	**Tesawa**	whitish brown	big	-	4-6
RC2	***C. arietinum***	**Tesawa**	white	moderate	-	4-6
RC3	***C. arietinum***	**Tesawa**	translucent	big	-	4-6
RA1	***Arachis hypogaea***	**Tesawa**	creamy	small	-	4-7
RVG1	***Vigna unguiculata***	**Tesawa**	brown	moderate	-	4-6
RVG2	***V. unguiculata***	**Tesawa**	brown	moderate	-	4-6
RVG3a	***V. unguiculata***	**Tesawa**	whitish brown	small	-	4-7
RVG3b	***V. unguiculata***	**Tesawa**	white	big	-	1-3
RVG4	***V. unguiculata***	**Tesawa**	translucent	small	-	4-7
RVG5	***V. unguiculata***	**Tesawa**	whitish creamy	big	-	1-3
RVG1wa	***V.unguiculata***	**Tesawa**	white	small	-	4-7
RVG1wb	***V. unguiculata***	**Tesawa**	white	big	-	1-3

RVG2w	***V. unguiculata***	**Tesawa**	white	moderate	-	4-6
RVG3wa	***V. unguiculata***	**Tesawa**	white	small	-	4-7
RVG3wb	***V. unguiculata***	**Tesawa**	white	big	-	1-3
RP4	***Pisum sativum***	**Tesawa**	whitish brown	small	-	4-7

Studies indicate that *Medicago* spp. is symbiotic with fast-growing rhizobia that belong to species of *Rhizobium* and *Ensifer* (Al-Idrissi et al., 1996; Trainer & Charles, 2006). *V. unguiculata* is symbiotic with both fast-growing and slow-growing rhizobia; however, it is slow-growing rhizobia are responsible for the nodulation (Young, 1992). *C. arietinum* is predominantly symbiotic with the *Mesorhizobium*, whose colonies are clearly visible after 72 h (Vincent, 1970). *Vicia faba* is symbiotic with the fast-growing species (Somasegaran & Hoben, 1994), while *L. varius* is symbiotic with both the fast-growing and slow-growing species (Howieson & Dilworth, 2016). Various Colors of colonies were previously uncommon in rhizobia, but recent studies have shown the ability of rhizobia colonies to appear with multiple colors; in Libya, such characteristics of rhizobia colonies have emerged in several studies involving cultivated and wild plants (Ahmadi, 2013). This trait and characteristics may help rhizobia to survive, and compete in the scarce environments.

The primary screening of the isolates and the optimal conditions for PHB production.

The results demonstrated that most of the isolates were producers of Polyhydroxybutyrate with diverse degrees (Table 2, Fig. 3). The appearance of the blue-black and the fluorescent color by the fluorescence microscope indicates the production of this compound.

Table 2. Production of Polyhydroxybutyrate according to the type of medium and under various conditions.

Isolates	**Production of PHB in different carbon and nitrogen sources**					**Medium2 with temperature**		**Medium2 with pH**	
	YEMA 1	**Medium 2**	**Medium 3**	**Medium 4**	**Medium 5**	**37°C**	**40°C**	**5pH**	**9pH**
R1	* -	+	-	-	-	+	+	+	+
R2	+	+	-	-	-	+	+	+	+
R3	+	+	-	-	-	+	+	+	+
R4a	-	+	-	-	-	+	+	+	+
R4b	-	+	-	-	-	+	+	+	+
R5	+	+	+	+	+	+	+	+	+
R7	-	+	-	-	-	+	+	+	+
R8	+	+	+	+	+	+	+	+	+
R9	+	+	+	+	+	+	+	+	+
R10	+	+	-	+	+	+	+	+	+
R11	+	+	-	-	-	+	+	+	+
R12	-	+	-	-	-	+	+	+	+
R13	+	+	-	-	-	+	+	+	+
R14	+	+	-	-	-	+	+	+	+
RVF1a	+	+	+	+	+	+	+	+	+
RVF1b	+++	+++	+++	+++	+++	+++	+++	++ +	++ +
RVF2	++	++	++	++	++	++	++	++	++

RVF4a	+	+	+	+	+	+	+	+	+
RVF4b	+	+	+	+	+	+	+	+	+
RVF5	+	+	+	+	+	+	+	+	+
RL	++	++	++	++	++	+	+	+	+
RC1a	++	++	++	++	++	+	+	+	+
RC1b	+++	+++	+++	+++	+++	+++	+++	+++	+++
RC2	++	++	++	++	++	++	++	++	++
RC3	+++	+++	+++	+++	+++	+++	+++	+++	+++
RA1	++	++	++	++	++	++	++	++	++
RVG1	+	+	+	+	+	+	+	+	+
RVG2	+	+	+	+	+	+	+	+	+
RVG3a	++	++	++	++	++	++	++	++	++
RVG3b	+	+	+	+	+	+	+	+	+
RVG4	±	+	+	+	+	+	+	+	+
RVG5	+++	+++	+++	+++	+++	+++	+++	+++	+++
RVG1wa	++	++	++	++	++	++	++	++	++
RVG1wb	++	++	++	++	++	++	++	++	++
RVG2w	++	++	++	++	++	++	++	++	++
RVG3wa	++	++	++	++	++	++	++	++	++
RVG3wb	++	++	++	++	++	++	++	++	++
RP4	±	-	-	-	-	-	-	-	-

*± Weak pigmentation, ++ Medium pigmentation, +++ Intense pigmentation, - Not pigmented.

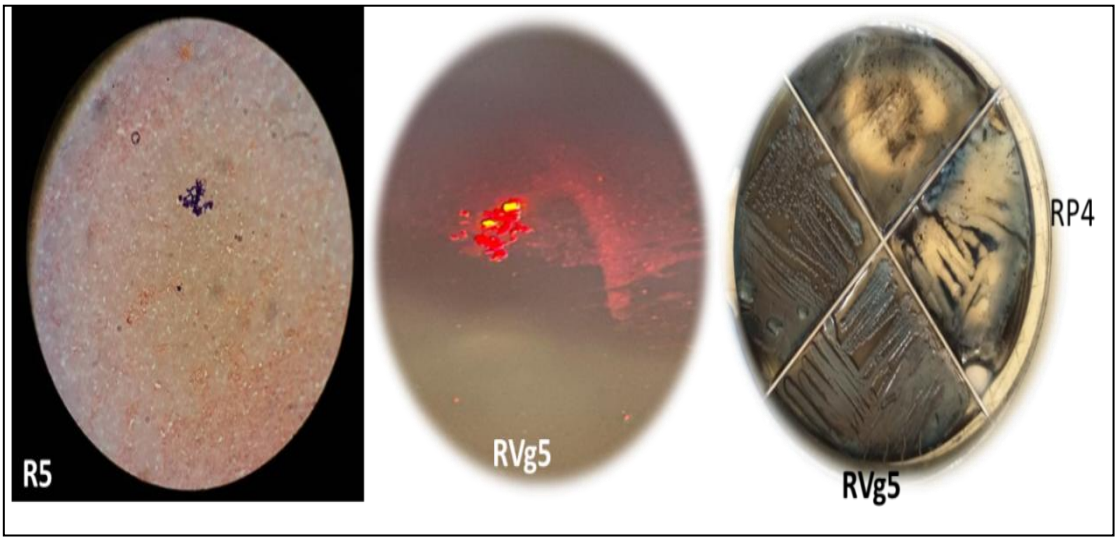

Fig. 3. Rhizobium isolates and their production of Polyhydroxybutyrate by plate immersion and glass slide methods.

PHB is found in many aerobic and facultative Gram-positive and Gram-negative bacteria such as *Bacillus*, *Escherichia*, *Pseudomonas*, *Azotobacter* and *Rhizobium*. Multiple Studies display

that bacteria have the ability to produce PHB with high efficiency, and its accumulation in the cell helps the bacteria to neutralize the acidity that is formed during the metabolic activity of the cell, and the cells utilize the PHB as a source of carbon and energy as well (Wang & Yu, 2000; Wang et al., 2007). The appearance of an intensive bluish-black color by some isolates with varying amounts of PHB depending on the strain which agree with what was reported by the others (Mercan et al., 2002; Wei et al., 2011). The isolates RVF1b, RC1b, RC3 and RVG5 were distinct compared to other isolates and were able to produce this Polyhydroxybutyrate in large quantities under all the tested conditions. The growth conditions of the microorganisms producing PHB were studied in order to find the best conditions that would lead to increasing its amounts. Through the obtained results, it can be said that there is a wide range of temperatures and pH could be optimal conditions for the releasing of PHB by the microbial isolates (Berlanga et al., 2006; Maheswari & Dhandayuthapani, 2013; Mahishi et al., 2003; Taepucharoen et al., 2017). In rhizobia, different types of carbon and nitrogen sources were used and all of them encouraged the manufacture of PHB (Mercan et al., 2002).

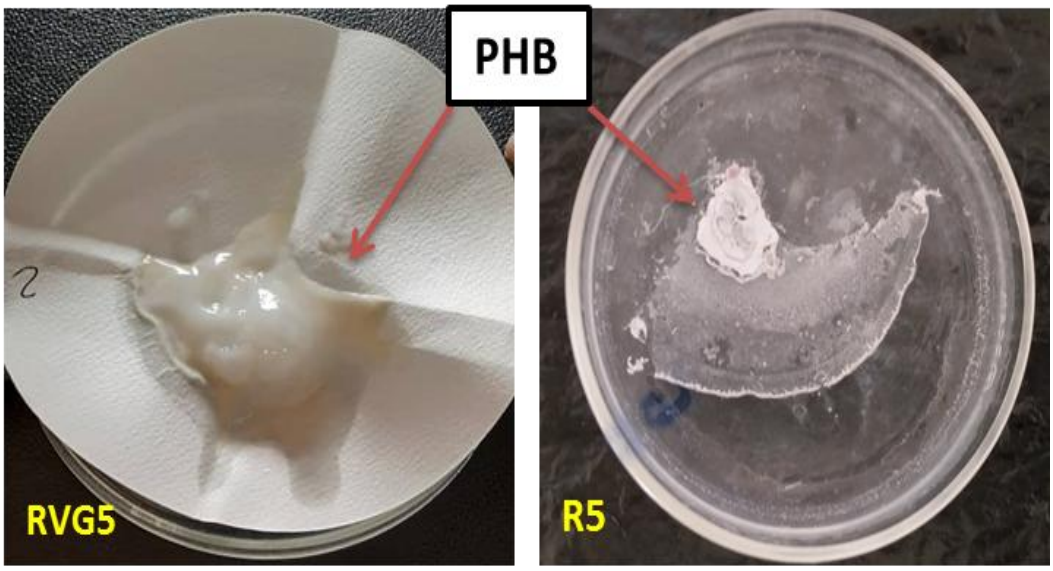

Fig. 4. Formation of a polyPHB membrane after extraction.

Studies indicate that PHB constitutes 30-50% of the dry cell weight in rhizobia (Wei et al., 2011). Studies also demonstrate that rhizobial strains vary in their efficiency in producing this polypeptide, from active to low efficiency, and this efficiency depends mainly on the carbon and nitrogen source in the environment (Mercan et al., 2002). The presence or absence of the gene responsible for the expression of PHB also plays a role in the production processes of the Polyhydroxybutyrate (Wang et al., 2007). PHB is stored as a carbon compound by a wide range of prokaryotes including rhizobia (Nair et al., 1993). They use it as a source of stored energy and as a redox regulator as well (Chandrashekharaiah, 2007; Macario, 2009).

The toxic effect of PHB

The results of the toxic effect of PHB that obtained from the isolate RVG5 (Fig. 5) showed that there was no an effect on the blood and no clotting occurred after incubation for 30 minutes; thus, the results agreed with many research as they mentioned that there was no a toxic effect of the PHB that was extracted from some microbes, including Cyanobacteria, and bacteria such as *Bacillus* (Abdo & Ali, 2018; Meixner et al., 2018).

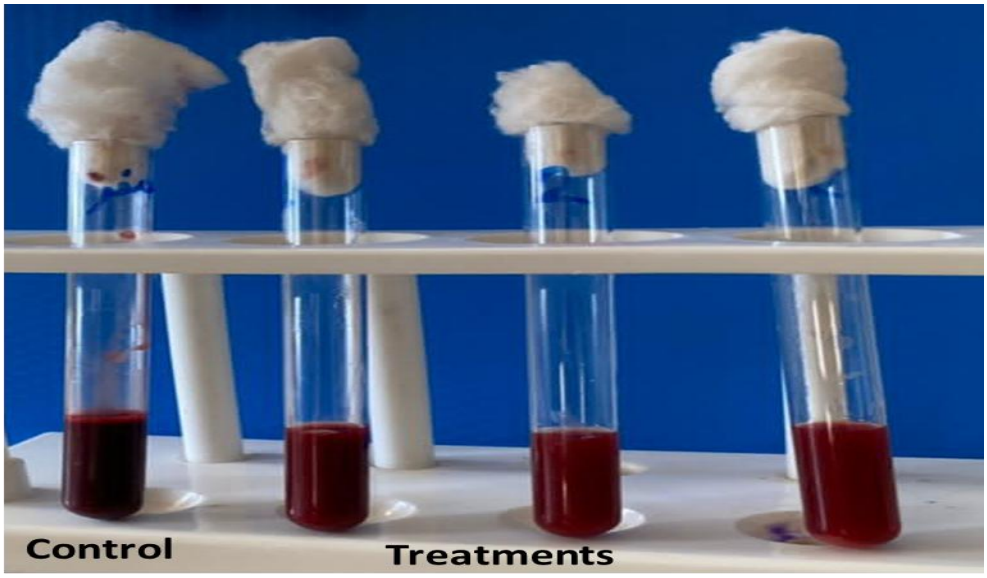

Fig. 5. Toxicity test of PHB produced from isolate RVG5.

The non-toxicity characteristic is what encouraged the use of this Polyhydroxybutyrate in many medical and food industries, as it is a natural, non-toxic, biodegradable material, and when it decomposes, it does not release toxic or environmentally harmful substances (Zinn et al., 2001).

Conclusion

This study confirmed that rhizobia associated with leguminous plants in the arid and semi-arid regions of Libya are capable of synthesizing poly-β-hydroxybutyrate (PHB), with clear variability in production potential among the isolates. The moderate- and slow-growing strains, particularly RVF1b, RC1b, RC3, and RVG5, consistently demonstrated superior PHB accumulation under different nutritional, temperature, and pH conditions. The extracted PHB showed no detectable toxicity to human blood, reinforcing its suitability for use in environmentally friendly and biomedical applications. These results highlight rhizobia as a promising microbial resource for PHB production and provide an important foundation for advancing local biopolymer biotechnology.

Disclaimer

The article has not been previously presented or published, and is not part of a thesis project.

Conflict of Interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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