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Original Research Article

Poly-β-hyroxybutyrate Production by Root Nodule Isolates of Wild *Vicia faba*

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ABSTRACT

Poly-β-hyroxybutyrate is a common bacterial polymer. It has been ecofriendly and best alternative biopolymer against environmental pollution. In this study, the poly-β-hyroxybutyrate (PHB) contents of *Rhizobium* isolates from isolated wild *Vicia faba* were studied in the media used by a spectrophotometric technique. The dry cell weights and PHB production of isolates were different. The production of PHB by 18 isolates ranged between 0.006-0.720 g/l with a productivity of 0.273-19.3 (%). B7 isolate produced more PHB than the other isolates.

Keywords: Poly-β-hydroxybutyrate, *Rhizobium*, isolate, wild *Vicia faba*

INTRODUCTION

The pastures are an important part of farming systems (Avcıoğlu *et al*. 2009). Şanlıurfa's natural pastures are approximately 234.527 ha and *Vicia, Trifolium, Pisum sativum, Vicia faba* are the primary legumes in natural pasture in Şanlıurfa, Turkey (Cevheri and Polat 2009). The soil bacterium *Rhizobium* sp. fixes nitrogen in symbiotic association with the forage legumes (Hansen 1994). *Rhizobium* bacteria have the exceptional ability to form nodules on roots of leguminous plants (Hansen 1994, O'Hara *et al.* 2003). The use of *Vicia faba* as a forage legume in Turkey required the introduction of the corresponding symbionts. The green and dry herbs, straw, silage, residues of *Vicia faba* evaluated for animal feed. Also, because it has quite a green hitch it can be used as green manure crops. One of the biggest feature of *Vicia faba* is the crude protein content reaching 30% in some varieties. The protein digested ratio and biological value due to tannins, lectins, glycosides some inhibitory substances is low (Avcıoğlu *et al.* 2009). The aim of the bean fodder production; as feed to make use of them. Also picks assessed before flowering or flowering process in order to meet the requirements of green feed ruminant animals (Cevheri and Polat 2009, Hansen 1994).

This species has wide adaptation and produces high quality forage under a grazing regimes (Avcıoğlu *et al.* 2009). Many papers have reported enhancement of nodulation and growth of a wide variety of forage and grain legumes by *Rhizobium* bacteria (Hansen 1994, Seguin *et al*. 2001, Zahran 2001, Khanna and Srivastava 2005). Symbiotic nitrogen fixation is often limited as a result of environmental conditions such as nutrient deficiencies, soil pH, temperature, soil moisture, salinity (Hansen 1994, Zahran 2001).

Poly-β-hydroxybutryrate is the the most common microbial biodegradeble plastics (Lee 1996**).** Poly-β-hydroxybutyrate (PHB) is synthesized under negative growth and environmental conditions by microorganisms such as bacteria, fungi and algae (Khanna and Srivastava 2005, Panigrahi and Badveli 2013). PHB are biodegradable polymers and they synthesized by numerous bacterial isolates such as root nodule bacteria (Anderson and Dawes 1990). Among these *Rhizobium* bacteria are *Rhizobium galega, R.heydsarum, R.leguminosarum, R.leguminosarum* biovar *trifolii, R.meliloti* (Tombilini and Nuti 1989, Tavernier *et al.* 1997).

These polymers are synthesized by *Rhizobium* bacteria when carbon source is abundant and growth is limited by the shortage of another nutrient (Zevenhuizen 1981). The purpose of this study was to isolate wild *Vicia faba* root nodule bacteria and determine PHB production by isolates.

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MATERIALS AND METHODS

Isolation

Rhizobium sp. isolates were isolated from root nodules of wild *Vicia faba* plants. For the isolation of *Rhizobium* sp. isolates from *Vicia faba;* uprooted roots having pink healthy nodules were selected, washed 5 times in distilled water and sterilized with 0.1% $HgCl_2$, crushed and growth in sterile yeast extract mannitol agar (Vincent 1970). Inoculated medium was incubated 28 ^oC for 5-6 days. The enriched sample was grown on congo red (0.0025 g/l of YEMA) containing Yeast Extract Mannitol Agar (YEMA) at 28 ^oC for 24 h. Isolates were purified and were tested for Gram reaction, colony structure, catalase, urease, growth at different pH levels, determination of NaCl tolerance, utilization of carbohydrates, hydrolysis of starch and gelatin (Jordan 1994) . Acid and alkali production was determined in YEMA medium with bromothymol blue indicator (0.0025 %) (Jordan 1994).

Analytic method for PHB

Determination of the amount of chemically. The cultures were inoculated with a 2% (v/v) inoculum, grown for 48 h in Yeast Extract Mannitol Broth at 250 rpm for 28 ^oC. At the end of this period, the samples were centrifuged for 20 min at 6000 rpm. The pellets were suspended in 5 ml sterile distilled water and then homogenized. 2 ml of the culture suspension was added 2 ml of 2 M HCl, the contents were heated at boiling temperature for 2 h in a water bath. Then, the samples were centrifuged at 6000 rpm for 20 min. Five millitre of chloroform were added to the resulting precipitate and the tubes were left overnight at 250 rpm at 28 ^oC. The contents were centrifuged at 6000 rpm for 20 min and extracted with 100 µl of chloroform, contents were dried at 60 ^oC. 10 ml 98 % sulpuric acid was added and heated at 60 ^oC for 1 h. The amount of PHB was measured at 235 nm in a UV spectrophotometer (Bonartseva and Myskina 1985, Bonartseva et al. 1994).

Also, cultures were grown at YEM broth, centrifuged at 6000 rpm for 30 min. The pellets were washed twice with sterile distilled water and dried for 24 h at 100 ^oC. The total bacterial dry weight was determined. All experiments were repeated three times. Their average values and Standard deviations (SD) are given.

RESULTS AND DISCUSSION

The bacteria isolated from the root nodules was identified as a *Rhizobium* sp. according to Bergey's Manual of Systematic Bacteriology (Jordan 1994) but the species was not ascertained. The bacteria were also identified following the methods given in the Manual of Microbiological Methods (Jordan 1994). The bacteria were rod shaped, aerobic, motile, gram negative and non-spore forming which showed positive results for catalase, acid production YEM agar, urease, citrate, nitrate reduction tests and could hydrolize gelatin. The bacterial isolates showed negative results in citrate utilization tests, 4% NaCl tolerance tests. The bacterial isolates produced acid in sugar medium and serum zone in litmus milk medium. All of the isolates gave negative results for strach hydrolysis, indole, voges Proskauer and methyl red tests (Table 1).

Also, isolates have shown tolerance at 1 %, 2% and 3 % NaCl. Similarly, variation of *Rhizobium* isolates subjected to NaCl tolerance, growth at different pH levels and carbohydrates is reported (Panigrahi and Badveli 2013, Jordan 1994, Junior *et al.* 2011).

In this study, PHB production of the root nodule isolates were detected. The cell dry weight and yield of PHB produced by root nodule bacterial isolates are shown in Table 2. The dry cell weights of cultures were different (Table 2). The production of PHB by 18 isolates ranged between 0.006-0.720 g/l with a productivity of 0.27-19.3 (%).

Table 1. Some morphological and biochemical characteristics of isolates.

Characteristics		Number of positive isolates	Number of negative isolates
Gram reaction		18	-
Cell shape		Short rods	
Growth at	15 °C	6	12
	30 °C	18	-
	37 °C	10	8
Growth at pH	5	18	-
	7	18	-
	8	15	3
Acid production on YEMA		18	-
Alkali production on YEMA		-	18
NaCl tolerance	1 %	18	-
	2%	18	-
	3%	18	-
Utilization of carbohydrates	Mannose, mannitol, xylose, raffinose, glucose, galactose, fructose, arabinose	18	-
	dulcitol	-	18
Catalase, nitrate, urease, citrate,		18	-
Indole, methyl red, Voges prokauer		-	18
Hydrolis	Starch	-	18
	Gelatin	18	-

Table 2. PHB production by root nodule isolates.

Isolates	Cell Dry Weight (g/l)	PHB (g/l)	Yield of PHB (%)[#]
B1	1.04 ± 0.04*	0.020 ± 0.003	1.92
B2	0.72 ± 0.110	0.016 ±0.015	2.22
B3	0.80 ± 0.005	0.218 ± 0.012	0.27
B4	0.79 ± 0.160	0.082 ±0.002	10.3
B5	0.27 ± 0.140	0.390 ±.0.004	1.44
B6	0.40 ± 0.03	0.445 ± 0.001	1.11
B7	1.18 ± 0.025	0.720 ± 0.021	6.10
B8	0.36 ± 0.035	0.006 ± 0.042	1.67
B9	0.65 ± 0.040	0.062 ± 0.006	9.53
B10	0.98 ± 0.050	0.013 ± 0.004	1.32
B11	0.72 ± 0.310	0.084 ± 0.005	11.7
B12	1.04 ± 0.025	0.092 ± 0.019	8.84
B13	0.57 ± 0.035	0.015 ± 0.015	2.63
B14	0.49 ± 0.050	0.095 ± 0.012	19.3**
B15	0.71 ± 0.620	0.017 ± 0.010	2.39
B16	0.65 ± 0.040	0.056 ± 0.018	8.61
B17	1.25 ± 0.052	0.050 ± 0.006	4
B18	1.17 ± 0.055	0.025 ± 0.001	2.13

*Values are the means ± Standard deviations of three measurements.

**The highest PHB production

[#]According to cell dry weight

B7 isolate produced more PHB than the other isolates and B8 produced less PHB than the other isolates did. In one of the the studies conducted by Hernandez *et al.* (2013), it was reported that *Rhizobium* isolates accumulated 0.001-0.009 g/l PHB during broth medium. The highest yield of PHB accumulation in comparison to dry weight was obtained in B14 isolate (19.3 %). Bergersen *et al.* (1991) reported that accumulate high levels of PHB during the active nitrogen fixing period of symbiosis of bean and soybean. Also, Rebah *et al.* (2009) reported that PHB yields (%) accumulated in cells according to dry weight were different; 7.27 % for *S.meliloti*; 5.23 % for *R.lupini*. Accumulated PHB in bacteroid can be mobilized for use during periods of low carbon availability (Rebah *et al.* 2009, Kretovich *et al.* 1977). Rebah *et al.* (2009), Lowing *et al.* (2005); Mercan *et al.* (2002) obtained that poly-β-hydroxybutyrates were important molecules in *Sinorhizobium meliloti, R.leguminosarum* bv. *phaseoli, Rhizobium leguminosarum* bv. *viciae.* PHB plays a role in cell protection during

stress periods such as drought, salt stresses, starvation and low nutrient availability (Tavernier *et al.* 1997, Tamdoğan and Sıdal 2011).

In this paper, the PHB production by *Rhizobium's* wild isolates were determinated in the medium. Further work will be carried out to interactions between nitrogenase activity and PHB production.

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