Egypt. J. Microbiol. **46,** pp.109- 123(2011)

Production of Polyhydroxyalkanoate (PHAs) and Copolymer [P(HB-co-HV)] by Soil Bacterial Isolates in Batch and Two-Stage Batch Cultures

Rawia F. Gamal, Hemmat M. Abd El-Hady, Enas A. Hassan, T. S. El-Tayeb and Khadiga A. Aboutaleb
Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima, Cairo, Egypt.

NINETY TWO local bacterial isolates, from the rhizosphere and soil around the root system of bean (*Vicia faba)* grown in Kalubia Governorate in Egypt, were bio-prospected for polyhydroxyalkanoate (PHA) accumulation. Three isolates accumulated ≥ 20 % of PHAs, they were identified as *Pseudomonas fluorescens* S48, *Bacillus megaterium* 7A and *B. megaterium* UBF19. The tested isolates gave the maximum PHAs content on basal medium containing glucose and ammonium sulfate at C/N ratio of 30/1 after 72 hr at 30^{o}C using shake flask culture technique. Two-stage batch were implemented with varying loading levels of nitrogen and phosphorus, inoculated with washed cells. Nitrogen omission of 70 % led to increase the PHAs content by 19 %, 3% and 8.5 % using washed cells of *Ps. fluorescens* S48, *B. megaterium* UBF19 and *Bacillus megaterium* 7A, respectively comparing with batch production on the same medium after 72 hr. The Copolymer poly(hydroxybutyrate-*co*-hydroxyvalerate) [P (HB-co-HV)] content level was increased when valeric/glucose (V/G) was 0.19 mol.mol^{-1} after 96 hr being 25.97 % and 20.11 % by *Ps. fluorescens* S48 and *B. megaterium* UBF19, respectively and reached 23.73 % by *B. megaterium* 7A at propionic/glucose (P/G) of 0.5 mol.mol^{-1}. The corresponding highest values of valeric content of copolymer at V/G 3.08 mol.mol^{-1} were 63 %, 49 % and 45 %, respectively,comparing with other V/G ratios by using GC analysis . Replacing glucose with 2 % corn oil or 1 % soybean oil increased the PHAs content of *Ps. fluorescens* S48 cells to 54.21 % and 52.12 %, respectively, after 72 hr.

Keywords: *Pseudomonas fluorescens*, Bioplastic, PHAs, [P(HB-co-HV)], Batch culture, Two-stage batch culture.

Polyhydroxyalkanoates (PHAs) are a class of natural polyesters, which can be produced and accumulated by many Gram-positive and Gram-negative bacteria from at least 75 different genera. These polymers are accumulated intracellularly under conditions of nutrient stress and act as a carbon and energy reserve (Steinbüchel, 2001 and Reddy *et al*., 2003). Bacterialy produced polyhydroxybutyrate and other PHA have sufficiently high molecular mass to

Contact authors e-mails: rawiagamal123@yahoo.com, enasath@hotmail.com

have polymer characteristics that are similar to conventional plastics such as polypropylene (Madison & Huisman, 1999). Poly-(3-hydroxybutyrate) (PHB) is the best-characterized PHA. Polymers of bacterial origin, such as polyhydroxyalkanoates (PHAs) are characterized as biocompatible "green" thermoplastic and are biodegraded in specific treatment systems (Galego *et al*., 2002). The chemical diversity of PHAs includes a wide spectrum of physical properties ranging from rigid, brittle plastics to softer plastics, elastomers, rubbers and glues (Poirier, 2002). The accumulation of intracellular storage polymers is another bacterial strategy that increases survival in a changing environment (Müller *et al*., 1999). Polyhydroxyalkanoates (PHA) serve as an endogenous source of carbon and energy during starvation (Kadouri *et al*., 2005). The isolated *Pseudomonas* sp. strain DR2 showed clear orange or red spots of accumulated PHA granules when grown on phosphate and nitrogen limited medium containing vegetable oil as the sole carbon source. Up to 37.34% (w/w) of intracellular PHA was produced from corn oil (Song *et al*., 2008). Also, Simon-Colin *et al*. (2008) investigated the biosynthesis of poly-(3-hydroxyalkanoates) (PHAs) by *Pseudomonas guezennei* using glucose and/or fatty acids with chain-length from 3 to 18 carbon atoms as carbon sources. Glucose, acetate, pyruvate, propionate, valerate, hexanoate, heptanoate, octanoate, decanoate, and oleic acid were supplemented in the mineral medium as the sole carbon source or as a mixture for PHAs accumulation. Under laboratory conditions, this bacterium produced a novel, medium-chain-length PHA, mainly composed of 3-hydroxydecanaote (64 mol. %) and 3-hydroxyoctanoate (24 mol. %) (GC-MS, NMR) from a single nonrelated carbon substrate, *i.e.*, glucose.

The present study examined the production of PHAs by different local bacteria under different culture conditions.

Materials and Methods

Soil samples for isolation of bacteria

Twenty soil samples were collected from the rhizosphere and the soil around the root system of bean plants (*Vicia faba*) grown in Kalubia Governorate, located in the Delta of the Nile. The soil samples were taken from 20 cm depth using pre-sterilized plastic scoops then put into sterile plastic bags and stored in iceboxes during their transport to the laboratory. In the laboratory all samples were kept refrigerated until isolation. The rhizosphere and soil samples collected from each plant were thoroughly mixed to compose representative samples. These soil samples were used for isolation of PHAs-accumulating bacteria.

Microbiological media used

Nutrient agar medium (Jacobs & Gerstein, 1960) was used for preservation of bacterial cultures. Kim *et al*. (1994) medium (basal medium) composed of (g/l): glucose, 10; $(NH_4)_2SO_4$, 1.0; KH_2PO_4 ,1.5; Na_2HPO_4. $12H_2O$. 9.0; $MgSO_4.7H_2O$, 0.2; pH 6.8 and 1 ml of trace elements solution ($FeSO_4.7H_2O$, 10; $ZnSO_4.7H_2O$,

2.25; $CuSO_4.5H_2O$, 1.0; $MnSO_4.4H_2O$, 0.5; $CaCl_2.2H_2O$, 2.0; $Na_2B_4O_7.10H_2O$, 0.23; $(NH_4)_6Mo_7O_{24}$, 0.1 and 35 % HCl 10 ml) and pH 6.8 was used for isolation of PHAs producing bacteria and as the productive medium.

Isolation and identification of PHAs producing bacteria

Serial dilutions up to 10^{-7} of each soil sample were prepared using sterilized water. Suitable dilutions were plated (in triplicates) on the above solid medium. After 4 days of incubation at 30°C, colonies were picked up, purified then maintained on nutrient agar at 4°C. PHAs producing bacteria were identified according the method described by Juan *et al.* (1998). The identification of the most efficient bacterial isolates was carried out according to Paul *et al.* (2004) and by the Biolog Microplate test panel (Biolog, Inc., USA) (Gelman *et al.,* 2000) at the Cairo Microbiological Resources Center (Cairo Mircen), Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Fermentation processes

In batch culture, the fermentation was carried out in 250 ml Erlenmeyer flasks containing 100 ml of basal medium, inoculated with 1 ml standard inoculum of the tested bacterial isolate ($5x10^8$ cfu/ml) which incubated at 30°C with shaking (150 rpm). Samples 10 ml were taken periodically to determine the cell dry weight (CDW) and PHAs content from triplicates shaken flasks and assayed in duplicates. In the two-stage batch culture, the first stage was carried out as previously mentioned in batch culture. After 24 hr of incubation, 10 ml was taken from the growing culture to estimate the CDW. Ninety ml remaining in the flasks was centrifuged at 10000 rpm and the sediment cells were used (non-washed and washed three times with sterilized distilled water) to inoculate the second stage flasks which contained 100 ml of the productive medium then incubated at 30°C for 96 hr. Samples were taken at different time intervals to determine the growth (CDW) and PHAs content during cultivation. Growth curves of the most effective PHAs producing bacteria (the relation between time (hr) and optical density at 620 nm was plotted). Specific growth rate (μ) and doubling time (t_d) were calculated from the exponential phase. Other fermentations were carried out as mentioned above except otherwise stated. All tests were performed in triplicates and the average was recorded.

Effect of medium components

In batch culture, growth and PHAs production were studied on different sugars as a sole carbon source *i.e*, galactose, fructose, mannose, xylose, sucrose and lactose instead of glucose as control. Effect of nitrogen sources *i.e*, tryptone, peptone, beef extract, yeast extract, NH_4Cl, $(NH_4)_2HPO_4$, $(NH_4)_2C_6H_6O_7$, NH_4NO_3 and $(NH_4)_2SO_4$ were tested. Different carbon and nitrogen sources were added equivalently to the original carbon and nitrogen percentages in basal medium. Different C/N ratios (17/1, 19/1, 23/1, 27/1, 30/1 and 31/1) were conducted using the most efficient carbon and nitrogen source obtained from previous optimization experiments.

Effect of different nutrient limitations in two-stage batch culture

Two sets of basal medium (Kim *et al*., 1994 medium) were used for the N and P limitation experiments. The first set included medium without ammonium sulfate as well as media containing 30 % and 40 % of nitrogen content. The second set included medium without phosphorus and media containing 37 % and 70 % of phosphorus content. The fermentation process was carried out as previously described in two-stage batch culture.

Effect of organic acids on copolymer P(HB-co-HV) production

This experiment was constructed in order to detect the proper concentration of either propionic or valeric acid in the productive medium, containing the distinctive carbon source to maximize the production of poly(hydroxybutyrate-*co*-hydroxyvalerate)P(HB-co-HV) by the selected isolates. Therefore, glucose (10 g/l) was replaced by propionic acid (2, 4, 6 and 8 ml/l) or valeric acid (1, 3, 5 and 7 ml/l) and completed with glucose in order to attain the original carbon concentration in the productive medium (10 g/l glucose). The ratios of propionic to glucose (P/G) and of valeric to glucose (V/G) were ranged between 0.5 – 5.163 and 0.19 – 3.08 $mol.mol^{-1}$, respectively. The cell density, polymer dry weight, propionic and valeric acid concentration (as mol %) were determined after 72 and 96 hr of incubation using shake flasks as a batch culture.

Effect of some vegetable oils on PHAs production

Five vegetable oils (olive oil, soybean oil, waste frying oil and corn oil) and oleic acid with three different concentrations (1, 2 and 3 %) were substituted with glucose in modified Kim *et al.* (1994) medium. Cell dry weight and final PHAs concentration were determined and PHA content % was calculated.

Determination of PHAs content

PHAs and total cell concentration was determined as dry weight (Grothe *et al*., 1999). The extraction of PHAs was implemented by the chloroform-sodium hypochlorite method (Hahn *et al*., 1994).

GC analysis of copolymer P(HB-co-HV)

Composition of copolymer P(HB-co-HV) (hydroxybutyrate & hydroxyvalerate) was determined by GC analysis as described by Mumtaz *et al.* (2009).

Statistical analysis

The collected data were statistically analysed using IBM® SPSS® Statistics software (2011).

Results and Discussion

Bioprospecting for polyhydroxyalkanoate (PHA) accumulating bacteria

Bioprospecting of polyhydroxyalkanoate accumulating bacteria isolated from the rhizosphere and soil apart of bean root system grown in Kalubia Governorate was carried out in an unbalanced medium (high carbon / nitrogen ratio) of Kim *et al.* (1994). Bacterial isolates were firstly screened for PHAs production depending on the test of Sudan Black staining. Colonies unable to

incorporate the Sudan Black appeared white, while PHAs producers appeared bluish black. Among ninety two local bacterial isolates, three isolates accumulated ≥ 20 % of PHAs after 96 hr of fermentation period at 30°C using shake flasks as a batch culture. These isolates were identified as *Ps. fluorescens* S48, *Bacillus megaterium* UBF19 and *B. megaterium* 7A**.**

Effect of carbon, nitrogen and C/N ratio on PHAs production in batch culture

The potentialities of *Ps. fluorescens* S48, *B. megaterium* UBF 19 and *B. megaterium* 7A as intracellular bioplastic polymer producers on six sources of carbon, nine sources of nitrogen and a range of C/N ratios were tested (Table 1). The highest polymer content (23 – 25 %) was attained in medium containing glucose and ammonium sulphate after 72 hr at 30°C. The corresponding figures of PHAs concentration were 0.35 – 0.402 g/l whereas the highest cell dry weight was attained in media containing peptone, beef extract and yeast extract by *Ps. fluorescens* S48, *B. megaterium* UBF 19 and *B. megaterium* 7A, respectively. Lee *et al*. (2000) reported that from the cultivation of *Anaerobiospirillum succiniproducens* and *Phaffia rhodozyma* in the presence of yeast extract and a combination of yeast extract and peptone to accumulate polyhydroxybutyrate (PHB). Khanna & Srivastava (2004) stated that using fructose and ammonium sulphate as a carbon and nitrogen source, *Alcaligenes eutrophus* exhibited a maximum biomass of 3.25 g/l with a PHB concentration 1.4 g/l after 48 hr of fermentation period. Regarding the effect of C/N ratio, 30/1 was the most proper, that the maximum PHAs accumulation increased from 23.11 % to 31.45 %, from 25.07 % to 30 % and from 23.13 % to 29.77 % for *Ps. fluorescens* S48, *B. megaterium* UBF19 and *B. megaterium* 7A, respectively.

From the previous results, it could be stated that the shortage of nitrogen source (ammonium) and the excess of carbon source (glucose) switched the cells from the growth mode to the accumulation mode (PHAs). The medium which contained 30/1 (C/N), will be called modified Kim *et al.* (1994) medium. Kanokphorn & Poonsuk (2008) mentioned that high (12/1) and low (<6/1) C/N ratio (mole C / mole N) caused significant decline of PHAs concentration (0.36 -3.67 g/l) and PHAs content (6.7 - 47.0 % of CDW) in *Rhodobacter sphaeroides* N20.

Time course of biomass production and PHAs accumulation

Data illustrated in Fig. 1 show that all the tested isolates grew exponentially during the first 4 – 24 hr (in modified Kim *et al*. (1994) medium 30/1 C/N) with specific growth rate (μ) ranged between 0.12 and 0.14 h^{-1} and doubling time (t_d) ranged between 4.95 and 5.79 hr. Time course analysis throughout 96 hr indicated that the polymer was growth associated and their accumulation started when cultures reached the stationary phase after 24 hr. The highest values of CDW were achieved after 54-60 hr of cultivation and then a slight decrease was coincided with a decrease in PHAs content. This result may be attributed to the decrease in the level of PHAs and PHB depolymerase due to nutrient depletion and cell consumption of PHB as a carbon source. On the contrary, Gjalt *et al.* (1989) stated that the fluorescent pseudomonads are characterized by their inability to make PHB, they appear to share the capacity to produce PHAs. This characteristic may be helpful in classifying pseudomonads. It may also be useful in the optimization of PHA production for biopolymer applications.

TABLE 1. Effect of different carbon sources, nitrogen sources and C/N ratios on the growth and PHAs production by *Ps. fluorescens* S48, *B. megaterium* UBF 19 and *B. megaterium* 7A in basal medium after 72 hr at 30°C using shake flasks as a batch culture.

Carbon sources	*Ps. fluorescens* S48			*Bacillus megaterium* UBF 19			*B. megaterium* 7A		
		PHAs			PHAs			PHAs	
	CDW (g/l)	Concentration (g/l)	Content (%)	CDW (g/l)	Concentration (g/l)	Content (%)	CDW (g/l)	Concentration (g/l)	Content (%)
Galactose	0.42	0.048	11.31	0.93	0.08	8.6	0.70	0.05	7.14
Fructose	1.56	0.259	16.6	1.33	0.25	18.8	1.76	0.347	19.72
Mannose	1.56	0.267	17.12	1.26	0.11	8.73	1.74	0.146	8.39
Xylose	1.40	0.183	13.07	1.36	0.08	5.88	1.62	0.139	8.58
Sucrose	1.43	0.174	12.17	1.1	0.15	13.64	1.92	0.376	19.58
Lactose	1.33	0.146	10.98	1.22	0.145	11.89	1.88	0.192	10.21
Glucose (control)	1.74	0.402	23.11	1.40	0.351	25.07	1.74	0.402	23.13
Mean	**1.36[b]**	**0.21[b]**	**14.92[a]**	**1.23[c]**	**0.17[c]**	**13.23[c]**	**1.62[a]**	**0.24[a]**	**3.82[b]**
Nitrogen sources									
Tryptone	1.08	0.064	5.90	0.70	0.09	12.86	1.10	0.210	19.09
Peptone	1.76	0.116	6.59	1.34	0.09	6.72	1.40	0.100	7.14
Beef extract	1.42	0.060	4.23	1.98	0.07	3.54	1.40	0.050	3.57
Yeast extract	1.46	0.307	21.03	1.68	0.05	2.98	2.10	0.328	15.62
NH_4Cl	1.04	0.070	6.73	1.20	0.10	8.33	1.26	0.250	19.84
$(NH_4)_2HPO_4$	1.16	0.058	5.00	1.06	0.05	4.72	1.30	0.060	4.62
$(NH_4)_2C_6H_6O$	1.14	0.040	3.51	1.48	0.06	4.05	1.46	0.196	13.42
$(NH_4NO_3$	1.46	0.090	6.16	1.62	0.07	4.32	1.38	0.150	10.87
$(NH_4)_2SO_4$ (control)	1.74	0.402	23.10	1.40	0.35	25.07	1.74	0.402	23.10
Mean	**1.36[b]**	**0.13[b]**	**9.15[b]**	**1.39[b]**	**0.11[a]**	**8.07[c]**	**1.48[a]**	**0.20[c]**	**13.04[a]**
C/N ratio									
17/1	1.34	0.287	21.42	1.05	0.175	16.67	1.39	0.292	21.01
19/1 (control)	1.22	0.282	23.11	1.40	0.351	25.07	1.31	0.303	23.13
23/1	1.12	0.300	26.79	1.55	0.377	24.32	1.24	0.301	24.27
27/1	1.20	0.356	29.67	1.61	0.440	27.33	1.16	0.305	26.29
30/1	1.24	0.39	31.45	1.26	0.378	30.00	1.30	0.387	29.77
31/1	0.26	0.068	26.15	0.58	0.169	29.41	0.22	0.043	19.55

CDW = Cell dry weight.

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

PHAs production using two-stage batch culture technique

Different nitrogen and phosphorus limitations were constructed using washed or non washed cells as inocula to the second-stage in order to increase the production of PHAs by the tested isolates. Using washed cells for all limitation treatments gave higher PHAs parameters comparing with non-washed cells. After 48 hr of incubation of the second-stage, increase polymer content was obtained using washed cells as inoculum for all the tested isolates grown on

modified Kim *et al*. (1994) after the omission of 70 % of nitrogen (Fig. 2). This treatment increased the content of PHAs by 19.24 %, 3.0 % and 8.5 % for *Ps. fluorescens* S48, *B. megaterium* UBF19 and *B. megaterium* 7A, respectively, comparing with batch production on modified Kim *et al*. (1994) after 72 hr (Table 1). The non-washed cells treatments had a negative impact either on biomass or polymer accumulation (data not shown). Results proved that a high PHAs content could be achieved after 48 hr of incubation in the second-stage, due to the high yield of polymer accumulated under nitrogen semi-starvation medium, inoculated with washed cells (stress conditions) of *Ps. fluorescens* S 48, *B. megaterium* UBF19 and *B. megaterium* 7A.

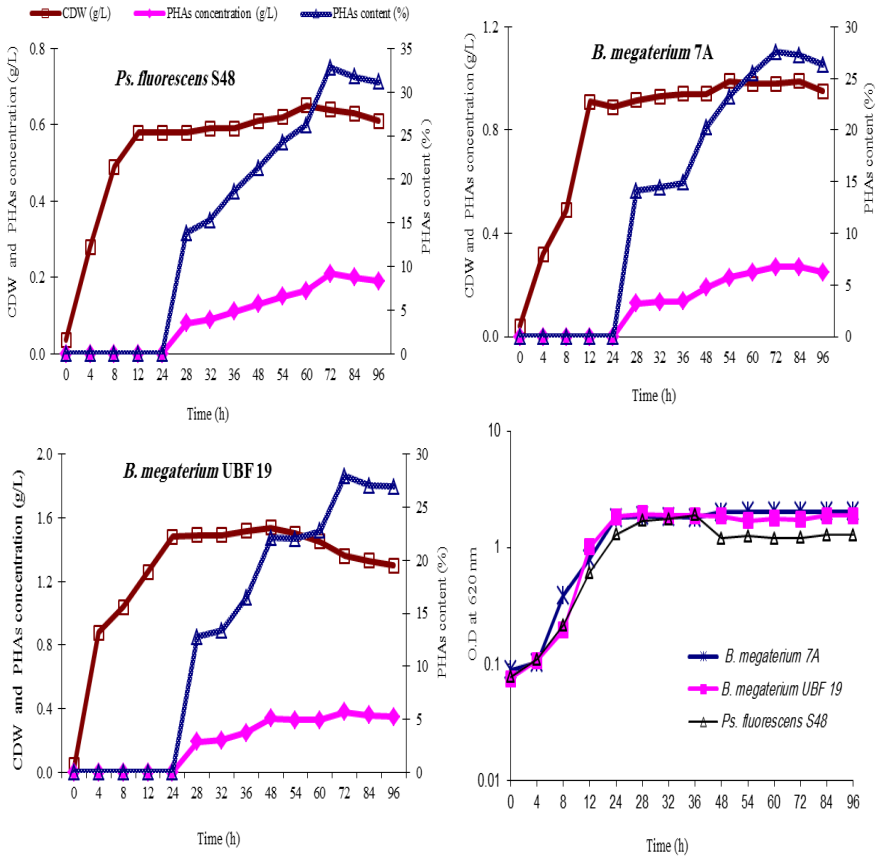


Fig. 1. PHAs production and growth curves of *Ps. fluorescens* S48, *B. megaterium* UBF 19 and *B. megaterium* 7A as affected by fermentation period at 30/1 C/N ratio medium using shake flasks as a batch culture.

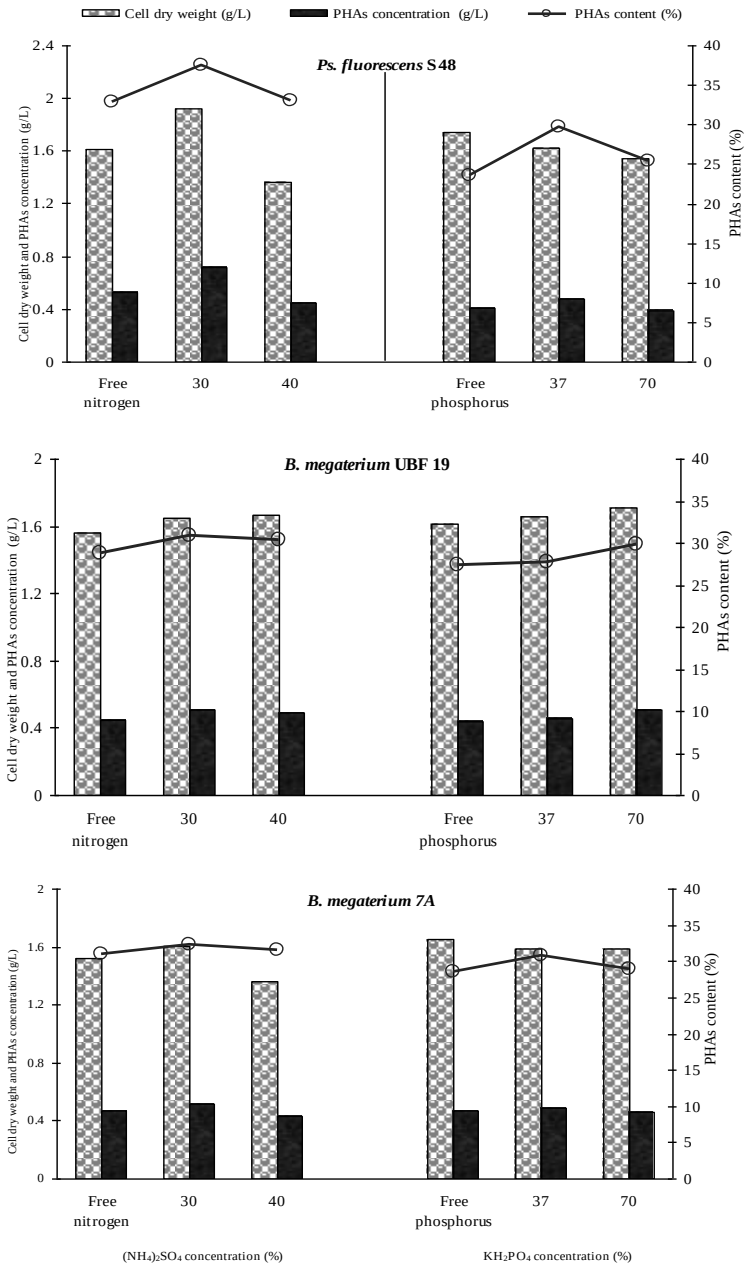


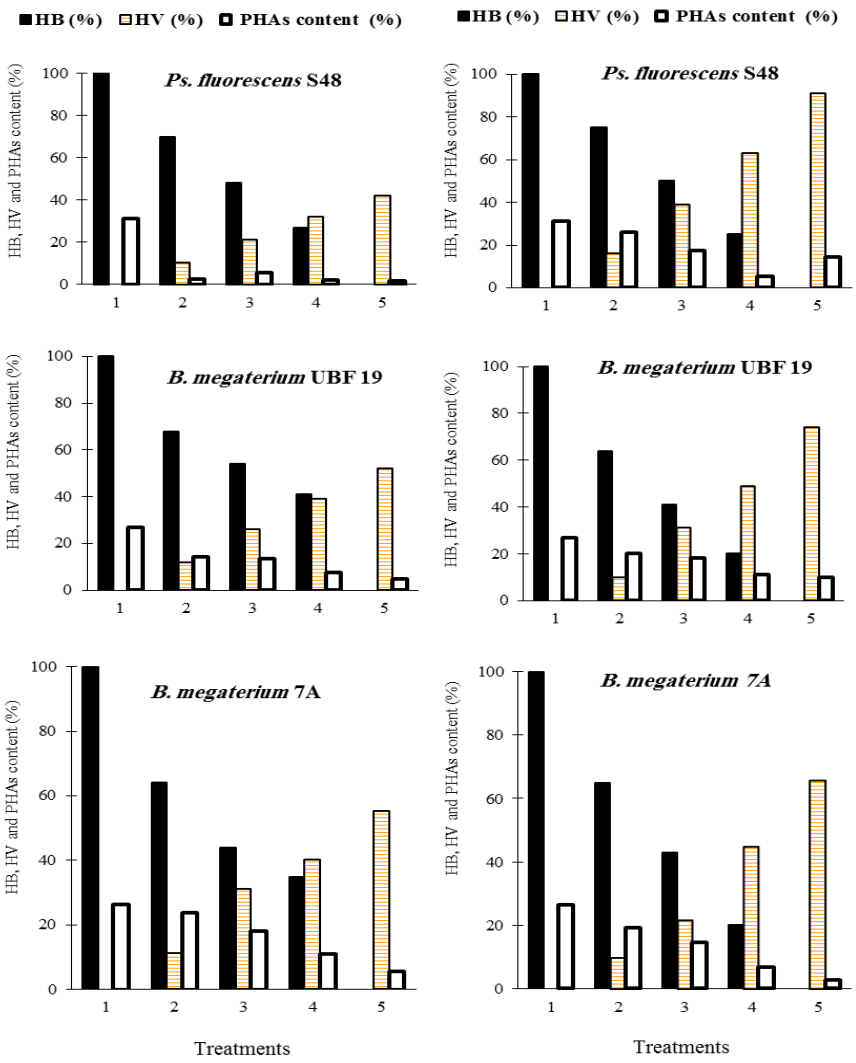
Fig. 2. Effect of nitrogen and phosphorus limitation treatments on polyhydroxyalkanoate production inoculated by washed cells of *Ps. fluorescens* S48, *B. megaterium* UBF 19 and *B. megaterium* 7A after 48 hr under second stage at 30°C using shake flasks as a two-stage batch culture.

With respect to phosphorus limitation treatments, data revealed that medium containing 37 % phosphorus was the best treatment in the accumulation of PHAs by *Ps. fluorescens* S 48 and *B. megaterium* 7A after 48 hr of the second-stage comparing with other treatments. Also it could be stated that increasing the incubation period of the second-stage to 72 hr didn't enhance the production of PHAs by the three tested isolates under different limitation treatments either using washed or non-washed cells. These results may be due to the capability of these bacteria to biodegrade PHB simultaneously under nitrogen or phosphorus limitation. Ramana (1996) detected this observation by *A. eutrophus* and called it "a cyclic nature of PHB metabolism". From the previous results it could be stated that the two-stage bioprocess is favorable for obtaining PHB than one-stage batch culture.

Effect of some organic acids on P(HB-co-HV) production in batch culture

Among the variety of PHA copolyesters, poly(3-hydroxybuyrate-co-3-hydroxyvalerate) copolyesters are of particular importance. It has a lower melting point and much better flexibility and has been commonly used in many applications (Yu *et al.,* 2005).

Data illustrated in Fig. 3 show that the highest values of copolymer content were obtained when V/G ratio was 0.19 (mol.mol^{-1}) after 96 hr (25.97 % and 20.11 % by *Ps. fluorescens* S48 and, *B. megaterium* UBF19, respectively). On the contrary, the proper ratio of organic acid to glucose was attained at 0.5 P/G (mol.mol^{-1}) under the same previous conditions by *B. megaterium* 7A (23.73 %). Also it could be stated that the addition of organic acids (P or V) to modified Kim *et al.* (1994) at any ratio decreased the content of copolymer concentration comparing with the control (glucose 10 g/l). This observation may be due to the toxicity of organic acid on biomass formation as well as on copolymer content and agreed with those obtained by Shimizu *et al*. (1999). GC analysis revealed that the polymer composition, *i.e.,* PHV / PHA ratio was increased with increasing P/G and V/G ratios. The highest HV content of total PHAs was 63 %, 49 % and 45 % by *Ps. fluorescens* S48, *B. megaterium* UBF19 and *B. megaterium* 7A, respectively with V/G 3.08 mol.mol^{-1}. On the other hand, the values of HV content were ranged from 10 to 32 %, 12 to 39 % and 11 to 40 % by *Ps. fluorescens* S48, *B. megaterium* UBF19 and *B. megaterium* 7A, respectively with P/G ratio ranged from 0.5 to 5.163 mol.mol^{-1}. Ramsay *et al*. (1990) noticed that HV content of total PHAs was 50 % by *B. cereus* and ranged from 30 to 45 % by *Pseudomonas* strains at proponic acid concentration ranging from 0.03 to 0.1 %. Serafim *et al*. (2001) stated that the HV % of total PHAs was 12 % by *Pseudomonas* strains with butyric acid feeding and was 10 % or 17 % by *B. cereus* with propionate or Heptanoate feeding, respectively (Valappil *et al*., 2006).



Treatment:
1- 10 g/l (Glucose)
2- 0.5 P/G ratio
3- 1.88 P/G ratio
4- 5.163 P/G ratio
5- 7.92 g/l (Propionic acid)

Treatment:
1- 10 g/l (Glucose)
2- 0.19 V/G ratio
3- 0.87 V/G ratio
4- 3.08 V/G ratio
5- 6.51g/l (Valeric acid)

Fig. 3. Effect of different ratios of propionic (P) or valeric acid (V) to glucose (mol.mol^{-1}) on copolymer [P(HB-co-HV)] content and composition produced by *Ps. fluorescens* S48, *B. megaterium* UBF 19 and *B. megaterium* 7A after 96hr at 30°C using shake flasks as a batch culture.

Effect of different oils

Since the data showed that *Ps. fluorescens* S48 was precede in PHAs accumulation than the other tested culture, in addition, many authors stated that plant oils seem to be the most suitable substrates because they are predicted to yield higher quantities of PHAs and subsequently reduce the cost of production by *Pseudomonas* sp. DR2, *P. fluorescens* PfO-1, *P. aureofaciens*, *Pseudomonas* sp.61-3 or *Pseudomonas oleovorans* (Halil *et al*., 2003; Karen *et al*., 2007; Nuttawee *et al*., 2004; Pettinari *et al*., 2001 and Loo & Kumar, 2007) . Therefore, this experiment was constructed to assess the feasibility of this approach. Glucose in modified Kim medium was substituted by three percentage levels of vegetables oil (olive , corn, soybean , waste frying oil) and oleic acid .

Data illustrated in Fig. 4 show that accumulated PHAs varied widely and depended on the kind of oil added to the medium. Soybean oil and corn oil could notably improve the biosynthesis of PHAs compared to glucose (control). The highest amount of PHAs was attained by adding 2 % of corn oil followed by 1 % of soybean after 72 hr (54.21 % and 52.12 %, respectively) using shake flasks as a batch culture. Therefore, it could be stated that using vegetable oils especially corn or soybean had a positive impact (1.7 fold of increase) on PHAs content by *Ps. fluorescens* S48, comparing with the control (glucose). In this respect, Karen *et al.* (2007) and Loo & Kumar (2007), stated that plant oils seem to be the most suitable substrates for PHAs production, because they are predicted to yield higher quantities of PHAs and subsequently reduce the cost of production by *Ps. fluorescens* PfO-1, or *Ps. Oleovorans.*

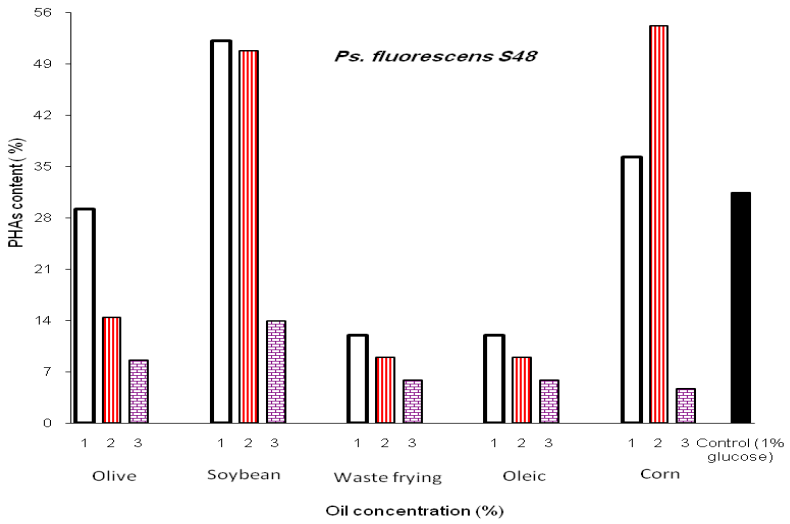


Fig. 4. PHAs production by *Ps. fluorescens* S48 as influenced by different concentrations of some vegetable oils and oleic acid after 72 hr of incubation at 30° C using shake flasks as a batch culture.

The further study involves the feasibility of using extracted oil from soybean meal,corn meal as well as types of waste frying oils to produce high content of PHAs using various fermentation process .

Acknowledgment: The authors are pleased to acknowledge Science and Technology Development Fund (STDF) for financial support.

References

Galego, N., Miguens, F.C. and Sanchez, R. (2002) Physical and functional characterization of PHASCL membranes. *Polym.* **43**, 3109–3114.

Gelman, A., Drabkin, V. and Larisa, G. (2000) Evaluation of lactic acid bacteria, isolated from lightly preserved fish products, as starter cultures for new fish-based food products. *Innov. Food Sci. Emerg. Technol.* 1, 219–226.

Gjalt, W.H., DeLeeuw, O., Eggink, G. and Witholt, B. (1989) Synthesis of polyhydroxyalkanoates is a common feature of Fluorescent Pseudomonads. *Appl. Environ. Microbiol*. **55**, 1949–1954.

Grothe, E., Moo-young, M. and Chisti, Y. (1999) Fermentation optimization for the production of poly (β–hydroxybutyric acid) microbial thermoplastic. *Enzym. Microb. Technol*. **25**,132–141.

Hahn, S.K., Chang, Y.K., Kim, B.S. and Chang, H.N. (1994) Optimization of microbial poly (3-hydroxybutarate) recovery using dispersions of sodium hypochlorite solution and chloroform. *Biotechnol. Bioeng.* **44**, 256–261.

Halil, K., Borcakli, M. and Demirel, S. (2003) Production of bacterial polyesters from some various new substrates by *Alcaligenes eutrophus* and *P. oleovorans*. *Turk. J. Chem.* **27,** 365-373.

IBM® SPSS® Statistics (2011) Version 19.0, SPSS Inc., Chicago, Illinois.

Jacobs, M.B. and Gerstein, M.J. (1960) ***"Handbook of Microbiology"***. Nostrand, D.V. (Ed.), pp. 139–202. Co., Inc., New York.

Juan, M.L., Gonzalez, L.W. and Walker, G.C. (1998) A novel screening method for isolating exopolysaccharide deficient mutants. *Appl. Environ. Microbiol.* **64**, 4600–4602.

Kadouri, D., Jurkevitch, E. and Okon, Y. (2005) Ecological and agricultural significance of bacterial polyhydroxyalkanoates. *Crit. Rev. Microbiol.* **31**, 55–67.

Kanokphorn, S. and Poonsuk, P. (2008) Nutrient optimization of PHB from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. *Electonic J. Biotechnol.* **11**, http://www.ejbiotechnology. info/content/ vol11/issue3/ full/2/2.pdf. Accessed (12/02/2011).

Karen, M.T., McGrath, J.W., Mullan, A., Quinn, J.P. and O'Connor, K.E. (2007) Polyphosphate accumulation by *P. putida* CA-3 and other medium–chain-length Polyhydroxyalkanoate-Accumulating bacteria under aerobic growth conditions. *Appl. Environ. Microbiol*. **73**, 1383–1387.

Khanna, S. and Srivastava, A.K. (2004) Recent advances in microbial polyhydroxy-alkanoates. *Process Biochem*. **40**, 607–619.

Kim, B.S., Lee, S.C., Lee, S.Y., Chang, H.N., Chang, Y.K. and Woo, S.I. (1994) Production of PHB acid by fed-batch culture of *Alcaligenes eutrophus* with glucose concentration control. *Biotechnol. Bioeng*. **43**, 892–898.

Lee, S.Y., Choi, J. and Lee, S.H. (2000) Production of polyhydroxyalkanoates by fermentation of bacteria. *Macromol. Symposia*. **159**, 259–266.

Loo, C.Y. and Kumar, S. (2007) Polyhydroxyalkanoates; Bio-based microbial plastics and their properties. *Malaysian Polym. J.* **2**, 31–57.

Madison, L.L. and Huisman, G.W. (1999) Metabolicengineering of poly (3-hydroxyalkanoates): from DNA to plastic. *Microbiol. Mol. Biol. Rev.* **63**, 21–53.

Müller, S., Bley, T. and Babel, W. (1999) Adaptative responses of *Alcaligenes eutrophus* to feast and famine conditions analysed by flow cytometry. *J. Biotechnol.* **75**, 81–97.

Mumtaz, T.O., Abd-Aziz, S., Abdul Rahman, N., Lai Yee, P., Shirai, Y. and Hassan, M.A. (2009) Fed-batch production of P (3HB-co-3HV) copolymer by *Comamonas* sp. EB 172 using mixed organic acids under dual nutrient limitation. *Eur. J. Sci. Res.* **33,** 374–384.

Nuttawee, N., Delamarre, S. C., Kim, Y. and Ba, C. A. (2004) Engineering of chimeric class II polyhydroxyalkanoate synthases. *Appl Environ Microbiol.* **70** (11), 6789–6799.

Paul, D.V., George, M.G., Dorothy, J., Fred, A.R., Karl-Heinz, S. and William, B.W. (2004) The firmicutes. In: "*Bergey's Manual of Systematic Bacteriology"*, Vol. 3, 2nd(ed.) de Vos, W.M. (Ed). pp. 1203–1259. Springer, New York.

Pettinari, M.J., Vázquez, G.J., Silberschmidt, D. and Rehm, B. (2001) Poly (3-hydroxybutarate) synthesis gene in *Azotobacter* sp. strain F A8. *Appl. Environ. Microbial*. **67** (11), 5331-53334.

Poirier, Y. (2002) Polyhydroxyalknoate synthesis in plants. *Prog. Lipid Res.* **41**, 131–155.

Ramana, C.H.V. (1996) Biodegradable polyesters. In: *"Advances in Applied Microbiology*", Volume 42, eidleman, S.L. and Laskin, A.I. (Ed.), p.97–218. Academic Press, California.

Ramsay, B.A., Lomaliza, K., Chavarie, C., Dube, B., Bataille, P. and Ramsay, J.A. (1990) Production of Poly-(β-hydroxybutyric-co-β-hydroxyvaleric) acids. *Appl. Environ. Microbiol.* **56**, 2093–2098.

Reddy, C.S.K., Ghai, R., Rashami, C. and Kalai, V.C. (2003) Polyhydroxyalkanoates: An overview. *Bioresour. Technol*. **87**, 137–146.

Serafim, L.S., Lemos, P.C., Ramos, A.M., Crespo, J.P. and Reis, M.A. (2001) Polyhydroxyalkanoates production by activated sludge. In: "*Biorelated Polymers: Sustainable Polymer Science and Technology"*, E.Chiellini, M. H.Mendes Gil, G.Braunegg, J.Buchert, P.Gatenholm, M.van der Zee, (Ed.), p. 147. Kluwer Academic Plenum Publishing, Dordrecht .

Shimizu, H., Kozaki, Y., Kodama, H. and Shioya, S. (1999) Maximum production strategy for biodegradable copolymer P(HB-co-HV) in fed-batch culture of *Alcaligenes eutrophus*. *Biotechnol. Bioeng*. **62**, 528–525.

Simon-Colin, C., Raguenes, G., Costa, B. and Guezennec, J. (2008) Biosynthesis of medium chain length poly-3-hydroxyalkanoates by *Pseudomonas guezennei* from various carbon sources. *React. Funct. Polym*. **68**, 1534–1541.

Song, J.H., Jeon, C.O., Choi, M.H., Yoon, S.C. and Park, W. (2008) Polyhydroxyalkanoate (PHA) production using waste vegetable oil by *Pseudomonas* sp. strain DR2. *J. Microbiol. Biotechnol.* **18**, 1408–15.

Steinbüchel, A. (2001) Perspectives for biotechnological production and utilization of biopolymers: metabolic engineering of polyhydroxyalkanoate biosynthesis pathways as a successful example. *Macromol. Biosci.* **1**, 1–24.

Valappil, S.P., Peiris, D., Langley, G.J., Herniman, J.M., Boccaccini, A.R., Bucke, C. and Roy, I. (2006) Polyhydroxyalkanoate (PHA) biosynthesis from structurally unrelated carbon sources by a newly characterized *Bacillus* spp. *J. Biotechnol.* **127**, 475–487.

Yu, S.T., Lin, C.C. and Too, J.R. (2005) PHBV production by *Ralstonia eutropha* in a continuous stirred tank reactor. *Process Biochemistry* **40,** 2729–2734.

(*Received* 14/4/2011;
accepted 5/10/2011)

إنتاج بولى هيدروكسي الكانويت (PHAs) و copolymer [P(HB-co-HV] من البكتيريا المحلية باستخدام طريقة المزرعة ذات الدفعة الواحده و طريقة المزرعة ذات المرحلتين

راوية فتحى جمال، همت محمد عبد الهادى، ايناس عبد التواب حسن ، طارق سعيد الطيب و خديجة أحمد أبو طالب
قسم الميكروبيولوجيا الزراعية - كلية الزراعة - جامعة عين شمس - شبرا الخيمة - القاهرة - مصر.

اثبتت نتائج التنقيب البيولوجى للبكتيريا التى لها القدرة على تكوين البولى هيدروكسي الكانويت و المعزوله من منطقة الجذور للفول البلدى بمحافظة القليوبية، ٩٢ عزله بكتيرية لها القدرة على انتاج البوليمر حيث تم الكشف باستخدام صبغه سودان بلاك. ثلاثة من هذه العزلات تحتوى على ٢٠ ٪ من البوليمر و تنتمى إلى *Pseudomonas fluorescens* S48 و *Bacillus megaterium* 7A و *B. megaterium* UBF19. و أعطت السلالات أعلى محتوى من PHAs على بيئة تحتوى على جلوكوز و كبريتات الأمونيوم بنسبه C/N (٣٠/١) بعد ٧٢ ساعة تحضين على ٣٠°م باستخدام طريقة المزرعة المهتزه (١٥٠ لفه فى الدقيقة). و تم إختبار طريقة المزرعة ذات المرحلتين تحت مستويات مختلفه من النيتروجين و الفوسفور فى البيئة الأصلية والتى تم تلقيحها بخلايا مغسولة أو غير مغسولة. واثبتت النتائج أن استبعاد ٧٠٪ من كمية النتيروجين ادى إلى زياده محتوى البوليمر بمقدار ١٩٪ و ٣٪ و ٥, ٨٪ باستخدام سلالات *Ps. fluorescens* S48 و *B. megaterium* UBF19 و *Bacillus megaterium* 7A المغسولة على التوالى. بينما التلقيح بالخلايا الغير مغسولة كان لها مردود سلبي سواء على الكتله الحيوية أو البوليمر. و سجلت أعلى نسبة للبوليمر ٢٥,٩٧٪ و ٢٠,١١٪ فى خلايا كلا من *Ps. fluorescens* S48 و *B. megaterium* UBF19 على التوالى عندما كانت نسبه حمض الفاليرك / الجلوكوز ٠,١٩ مول/مول بعد ٩٦ ساعـة من التحضين على ٣٠°م بينما وصلت إلى ٢٣, ٧٣٪ بواسطة *Bacillus megaterium* 7A فى الخلايا الناتجة من النمو على البيئة التى تحتوى على نسبة حمض البروبيونيك/جلوكوز ٠,٥ مول/مول. و كانت اعلى قيم لمحتوى الفاليرك فى البوليمر٦٣٪ و ٤٩٪ و ٤٥٪ بواسطه *Ps. fluorescens* S48 و *B. megaterium* UBF19 و *Bacillus megaterium* 7A على التوالى باستخدام نسبة حمض V/G ٣,٠٨ مول/مول بعد ٩٦ ساعة. و باستبدال الجلوكوز بواسطه ٢٪ زيت الذره أو ١٪ زيت فول صويا ادى إلى زياده محتوى البوليمر لـــــــــ ٥٤,٢١ ٪ و ٥٢,١٢ ٪ بخلايا *Ps. fluorescens* S48 بعد ٧٢ ساعة من التحضين على التوالى باستخدام طريقة المزرعة ذات الدفعة الواحده.