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ADHESION OF AMYLOLYTIC BACTERIA ON STARCH-POLYETHYLENE PLASTIC FILMS

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ABSTRACT

A comparative study of the adhesion ($\mu g\ cm^{-2}$) of Pseudomonas stutzeri and Peanibacillus polymyxa on starch-polyethylene films was made. Incubation of plastic films was carried out at room temperature for 40 days, in sediment and compost samples, respectively. The increase in weight of incubated and washed plastic films was determined in four replica. Adhesion of the tested bacteria on different starch-polyethylene plastic films was examined under the electron microscope. The amylase activity of the two bacterial strains isolated from compost (Peanibacillus polymyxa) and sediment (Pseudomonas stutzeri) was detected in pure and mixed cultures. The maximum specific amylase activity of the amylolytic bacteria Peanibacillus polymyxa was 1.45-fold higher than that of Pseudomonas stutzeri while mixed cultures of both bacteria showed no significant difference ($p<0.05$) comparing with the amylase activity produced by Peanibacillus polymyxa . The different starch concentrations (0.0, 10.0 and 20.0 g l) showed a significant effect ($p<0.05$) on the amylase activity of the tested cultures.

INTRODUCTION

The possible production of plastic materials containing substantial levels of starch as co-polymer instead of more traditional, petroleum-derived polymers, have been developed, e.g. Starch-polymethyl acrylate (PMA) (Swanson, *et. al.*, 1984), as well as simple mixtures of conventional hydrocarbon polymers,

polyethylene (PE) and polyethylene co-acrylic acid (EAA) (Otey, *et .al.*, 1987). Moreover, Lee *et.al.*(1991) reported that the most commonly suggested uses for starch-based degradable plastics is for composting of lawn, garden and shrub litter. Therefore, the volume of material entering the landfills could be reduced up to 20%. They also investigated the ability of litter- or lignocellulose-degrading microorganisms to attack starch-containing degradable plastics in pure culture.

Imam and Gould (1990) reported that the ability of microorganisms to adhere to the surface of insoluble substances is critical for their survival in the environment because of intense competition for limited resources. The fate of biodegradable plastics depends on how successfully amylolytic microorganisms are able to colonize their surface. Anderson and Salyers (1989) showed that starch breakdown by *Bacteroides thetaiataomicron* occurred by enzymes which are not secreted extracellularly and the binding of the bacterial cell surface to the starch molecule appears to be the first step in the biodegradation process. While Aguilar *et al.* (2000) found that, the extracellular α-amylases produced by *Lactobacillus manihotivorans* showed little activity when soluble starch was used as a substrate.

Several microorganisms were reported. for the production of amylases from different natural sources, e.g., β-amylases from sweet potato (Teotia, *et al.*, 2001), α-amylaes by *Penicillium expansum* and *Aspergillus oryzae* through the degradation of maltolignosaccharides (Doyle, *et al.*, 1999).

In this work, it is aimed to compare between the ability of local bacterial species to adhere on different starch-polyethylene plastic films as a first step for their degradation in the environment. The amylase activity of two isolated amylolytic bacteria (*Pseudomonas stutzeri* and *Peanibacillus polymyxa*) was estimated in presence of different starch concentrations.

MATERIALS AND METHODS

Organisms and cultivation

The bacterial strains were previously isolated and identified as *Pseudomonas stutzeri* and *Peanibacillus polymyxa* (El-Naggar and El-Aassar, 2000). Stock cultures were maintained on starch-nitrate agar medium.

Cultivation for amylase production was carried out in 250 ml Erlenmeyer flasks each containing 100 ml of starch-nitrate medium: $NaNo_3$ 2.0 g/l; K_2HPO_4 1.0 g/l; $MgSO_4$, $7H_2O$ 0.5 g/l; $FeSO_4$. $7H_2O$ 0.001 g/l and 1gm pieces of a mixture of different starch-polyethylene films (rice : corn : potato in a ratio of 1:1:1). Different soluble corn starch concentrations were added to study their effect on growth and amylase activity (0.0, 10 or 20 g/l). The medium was adjusted at pH 7.0 ± 0.2, each flask was inoculated with 3ml of bacterial suspension from 24 h culture. Cultivation was carried out under shaken conditions (160 rpm) at 37°C.

Plastics

The plastic films were provided from plastic development center, Alexandria, Egypt. Three different available starch granules (rice, corn and potato) were used in different concentrations for the manufacture of these plastic films. The strips were cut in the transverse direction to the blowing direction of the film, with 1.5 cm in width, 10 cm in length and the thickness was 0.02-0.05 mm.

Adhesion of the bacterial community

Each plastic strip was washed with alcohol and sterile distilled water, dried in an oven at 40-50°C for 1-2 h and weighed (W_1).

These strips (5 strips/dish) were incubated for 40 days separately in sterile petri dishes, 16cm in diameter, containing 40-50 gm of sterile marine sediment or compost samples. These samples were inoculated with 10ml cell suspension (10^7 cell/ml) of *Pseudomonas stutzeri* and *Peanibacillus polymyxa.* respectively. Then each strip was submersed and washed several times using sterile distilled water to remove all holding residues. Repeat the drying and weight determination process (W_2).

The difference in weight ($W_2 - W_1$) was used in relation to the total surface area (A) of the plastic strip under test as an indication for the relative microbial adhesion ($W_2 - W_1 / A$).

Scanning electron microscope

For scanning electron microscopy analysis, portions of the tested plastic strips were fixed in 1% glutarldehyd, dehydrated for 10 min, each in 50, 75, 90 and 100% ethanol and mounted on aluminum stabs. Samples were sputter

coated with gold using JEOL- JFC-1100E Ion sputtering device. Then visualized under a JEOL scanning microscope JSM-5300.

Light microscope examination:

For microscopic examination a light microscope fixed with camera was used. It made by Reichert Jung photo star USA, with the following specifications, model microstar IV, low magnification lenses (40x) (Reichert USA 1745-100ph) and high magnification lenses (400x) (Reichert USA 1742-10ph).

Assay for amylolytic activity

The amylase activity was determined as indicated by Bregman *et al.* (1988). 0.2 ml of culture supernatant was added to 0.1 ml of 0.5% saline starch in 0.2 M phosphate buffer (pH 7.1). Similar reaction mixtures using heated inactive enzyme solution were also prepared as control. The reaction mixture was incubated in a water bath at 37°C for 30 min. The released reducing sugars were measured using 1% dinitrosalycilic acid reagent (Moller, 1959). One unit of enzyme activity was defined as the amount of enzyme able to liberate 1µ mole maltose /min under the conditions mentioned before.

Estimation of protein

The total soluble proteins produced in the culture supernatant were determined according to Lowry *et al.* (1951) and modified by Tsuyosh and James (1978) using bovine serum albumin as a standard. The specific activity of the enzyme was calculated in U/mg protein.

Determination of growth

The growth of the tested bacterial cells was detected by measuring the dry weight along the incubation period (8 days). The dry weights were estimated after centrifugation at 6000 rpm for 15 min., washing twice with distilled water and then drying in a small pre-weighted aluminum foil dish for 24 h at 70°C.

RESULTS

From the results of the relative adhesion of the amylolytic, bacterial cells (Table 1), it was found that when the biodegradable plastic strips were incubated in the sediment samples (*Pseudomonas stutzeri)*, there was a significant difference ($P<0.05$) in the bacterial adhesion between plastic strips containing corn-starch at concentrations of 2 or 3% and the control strips

Table (1): The relative adhesion of the amylolytic bacteria on different starch-polyethylene plastic films before and after an incubation period of 40 days in sediment and compost samples.

Type of starch	Starch conc. (%)	*Pseudomonas stutzeri*			*Peanibacillus polymyxa*		
		Original Wt. (mg)	Wt. After Incubation (mg)	Relative adhesion @ (µg/ cm^2)	Original Wt. (mg)	Wt. after incubation (mg)	Relative adhesion @ (µg/ cm^2)
Control	0	94.7	95.3	21.6 ± 1.9^{a}	100.5	101.1	16.6 ± 0.17^{a}
Rice starch	2.5	97.2	102.2	$131.6\pm 11.9^{b**}$	94.3	97.8	$116.6\pm 13.1^{bc*}$
	5	97.4	105.0	$255.0\pm 9.6^{f**}$	71.7	74.8	$101.6\pm 5.8^{b*}$
Corn starch	2	71.5	73.2	$56.7\pm 10.5^{b*}$	72.8	75.9	$103.4\pm 13.0^{b*}$
	3	90.7	92.8	$70.8\pm 8.3^{b*}$	92.6	98.4	$195.0\pm 27.9^{cd*}$
	4	74.5	78.0	$115.8\pm 11.9^{c**}$	69.1	74.4	$175.8\pm 1.7^{c*}$
Potato starch	2	69.9	74.8	$164.2\pm 8.8^{d**}$	68.1	70.4	$75.0\pm 5.7^{b*}$
	3	64.6	70.6	$200.8\pm 9.6^{e**}$	68.7	73.2	$150.8\pm 21.6^{c**}$
	4	74.51	81.6	$237.5\pm 6.2^{f**}$	70.3	77.1	$225.0\pm 10.4^{d**}$
	10	97.7	101.7	$135.0\pm 10.4^{e**}$	96.6	99.7	$103.3\pm 7.2^{b*}$

$LSD_{0.05}$ for sediment = 25.00 LSD 0.01 for sediment = 85.7

$LSD_{0.05}$ for compost = 45.8 L SD 0.01 for compost = 182.2

Means present in the same column and have the same superscript are not significantly different ($p<0.05$).

*Significant difference ($p<0.05$) ** Highly significant ($P<0.01$) compared with the control.

@(values are given as a mean of four readings ± SD)

(starch-free polyethylene, PE). While adhesion of the tested bacteria on plastic strips containing different starch (rice or potato) or 4% corn starch concentrations showed a highly significant difference ($P<0.01$) when compared to adhesion of starch-free polyethylene used. It was noticed that a maximum bacterial mass adhesion values reaching 255.0 $\mu g/cm^2$ and 237.0 $\mu g/cm^2$ were obtained when 5% rice-starch and 4% potato starch were used in the manufacture of these plastic films, respectively.

The incubation of the tested starch-polyethylene plastic films in the compost samples (*Peanibacillus polymyxa*) showed a significant difference in the relative adhesion regardless to the type or the concentration of starch used compared with the control. A highly significant difference ($P< 0.01$) was obtained when 4% potato starch was used ($225 \mu g/cm^2$) compared with the control (starch-free polyethylene).

Distribution of starch granules and adhesion of bacterial cells on the control plastic films as well as starch treated polyethylene plastic films was observed by light and electron microscope. In figure 1 and 2 , A,C,E and G show the light microscope photographs (400x) of the starch-free and starch- polyethylene plastic films. The distribution, shape and size of rice, corn and potato starch granules was observed, respectively. It is noticed that the starch granules vary in shape from highly angular in rice and corn to round and oval shaped in potato.

In figure 1 and 2, B,D, F and H, show the adhesion of amylolytic bacteria *Peanibacillus polymyxa* and *Pseudomonas stutzeri* on the tested plastic films, observed by the scanning electron microscope. It was found that, the bacterial adhesion varied depending on distribution, size, shape and the type of the starch granules in the plastic films. Nearly no adhesion was observed on starch free polyethylene films (PE), while a significant adhesion of bacterial cells was observed on the starch containing plastic films.

The production of amylase activity by the tested bacteria was examined in cultures containing 1g pieces of starch-polyethylene plastic films only as well as in cultures supplied with soluble corn starch to enhance growth and activity. The results in table 2 showed that, the type of the used culture of *P.stutzeri* and *P.polymyxa* had no significant effect ($P< 0.05$) on the amylase production at each starch concentration during the maximum enzyme production period.

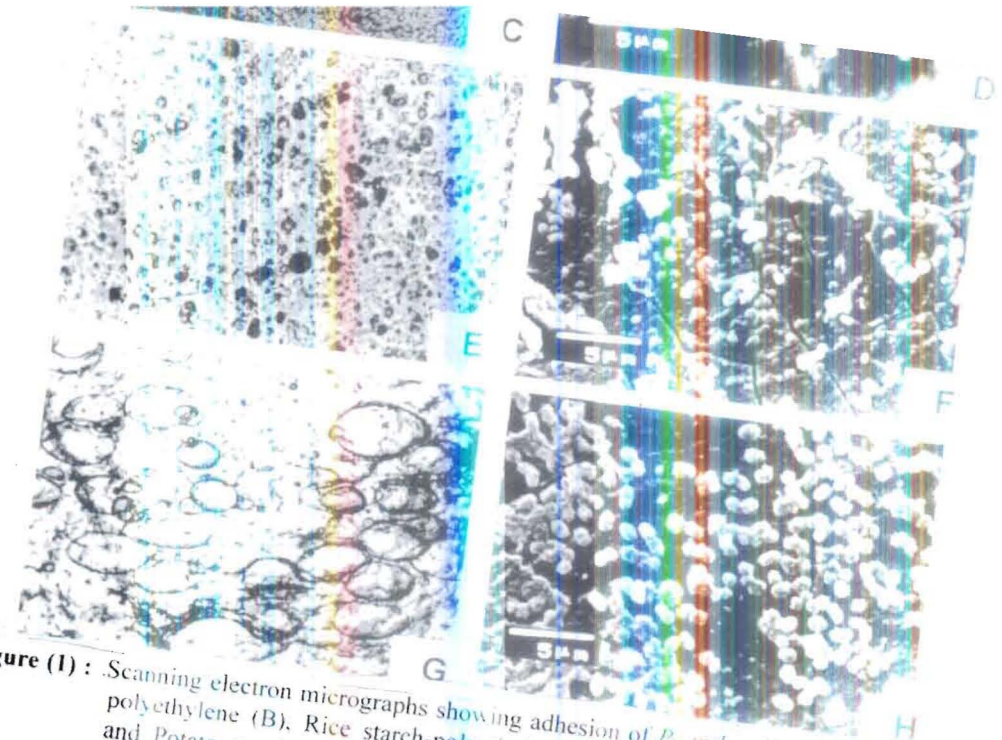

Figure (1) : Scanning electron micrographs showing adhesion of *Paenibacillus polymyxa* to polyethylene (B), Rice starch-polyethylene (D),Corn starch-polyethylene (F) and Potato starch- polyethylene (H) Corresponding non-inoculated controls examined by light microscope (400x) , showing the distribution of various starch granules on these polyethylene plastic films A,C,E and G , top to bottom

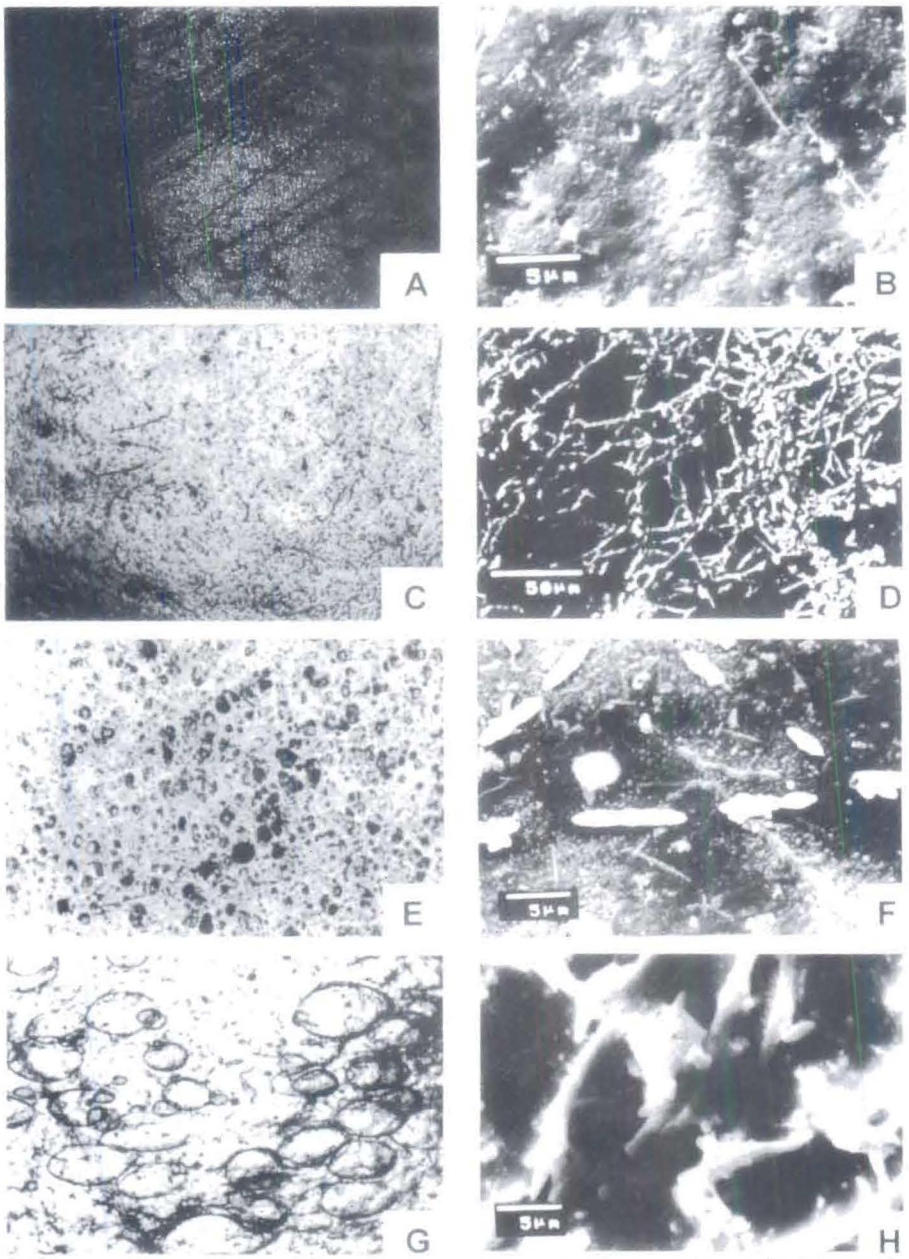

Figure (2) : Scanning electron micrographs showing adhesion of *Pseudomonas stutzeri* to polyethylene (B), Rice starch-polyethylene (D),Corn starch-polyethylene (F) and Potato starch- polyethylene (H). Corresponding non-inoculated controls examined by light microscope (400x) , showing the distribution of various starch granules on these polyethylene plastic films A,C,E and G , top to bottom

However, the highest value of activity obtained by *P.polymyxa* showed a significant difference when compared with that of *P.stutzeri*. It was also noticed the use of different starch concentrations (0.0, 10.0 and 20.0 g/l) showed a significant difference ($P< 0.05$) on the amylase produced by each cultures. Amylase activity was estimated in pure and mixed cultures of *Pseudomonas stutzeri* and *Peanibacillus polymyxa* over an incubation period of 8 days (Figure 3). A maximum enzyme production was obtained after 3 days of incubation using 20g/l starch concentration reaching a specific activity of 57.6, 83.8 and 84.8 U/mg protein in A,B and C, respectively.

The effect of the different starch concentrations (0.0, 10.0 and 20.0 g/l) on the growth of *Peanibacillus polymyxa* and *Pseudomonas stutzeri* cultures was detected (Figure 4). It was observed that, the dry weight of the tested bacteria directly increased with increasing starch concentration in the medium. The maximum values of the dry weight of *P.stutzeri* and *P.polymyxa* were obtained after 3 days of incubation reaching 385.7 and 483.9 mg/100 ml, respectively. While the dry weight of the mixed culture of these bacteria was 475.2 mg/100ml showing insignificant difference when compared to the dry weight of *Peanibacillus polymyxa*.

Table (2): The specific activity of the amylase enzyme produced by pure and mixed culture of *Pseudomonas stutzeri* and *Peanibacillus polymyxa* during the maximum enzyme production period.

Starch conc. (g/l)	Amylase activity (U/mg protein)			
	P. stutzeri	*P. polymyxa*	*P. stutzeri* & *P. polymyxa*	LSD (P< 0.05)
0-0	0.36±0.08[a]	1.07±0.55[a]	1.67±1.3[a]	0.671
10.0	13.25±5.7[b]	18.8±7.7[b]	32.99±9.7[b]	15.8
20.0	49.25±9.1[c]	75.21±9.4[c]	70.96±17.1[c]	N.S*
LSD (P< 0.05)	9.2	17.3	20.1	

Means presented by different supercript are significantly different ($P< 0.05$)
(Values are given as a mean of three readings ± SD)
* N.S : not significant

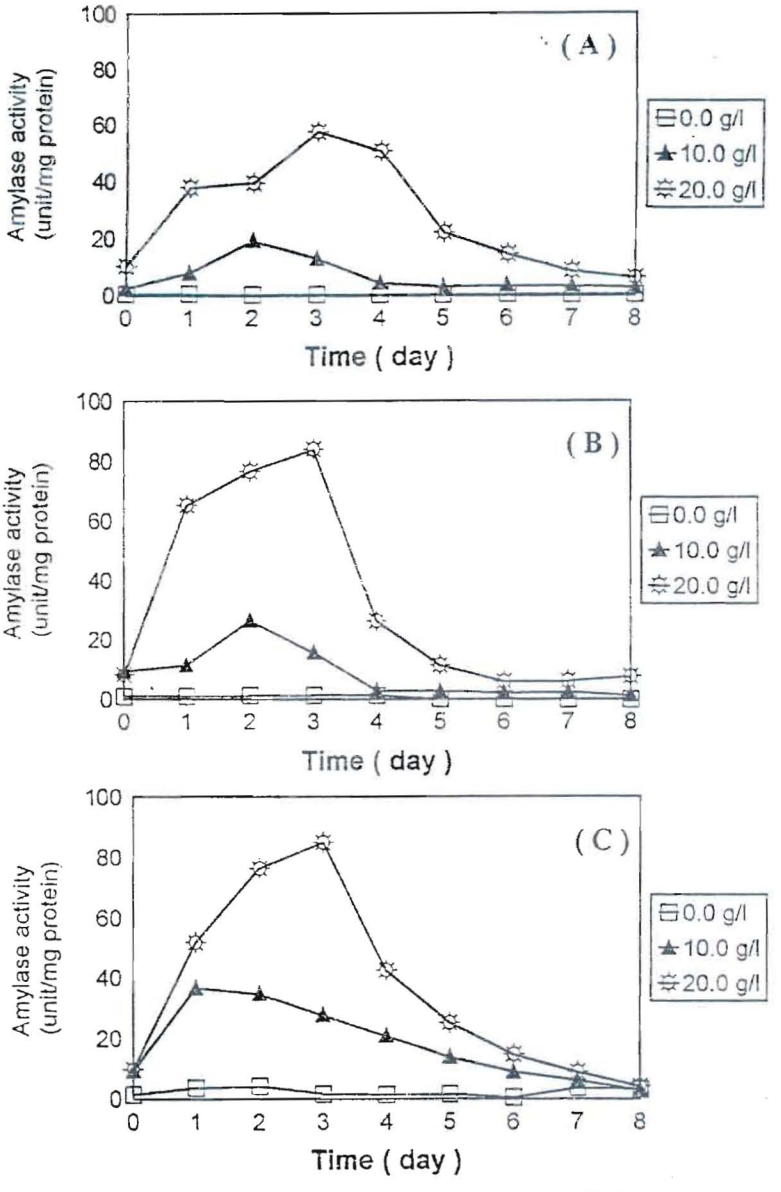

Figure (3) : Effect of different starch concentrations (0.0,10.0 and 20.0 g/l) on the specific activity of amylase produced by *Pseudomonas stutzeri* (A), *Peanibacillus polymyxa* (B) and by a mixed culture of A & B (C).

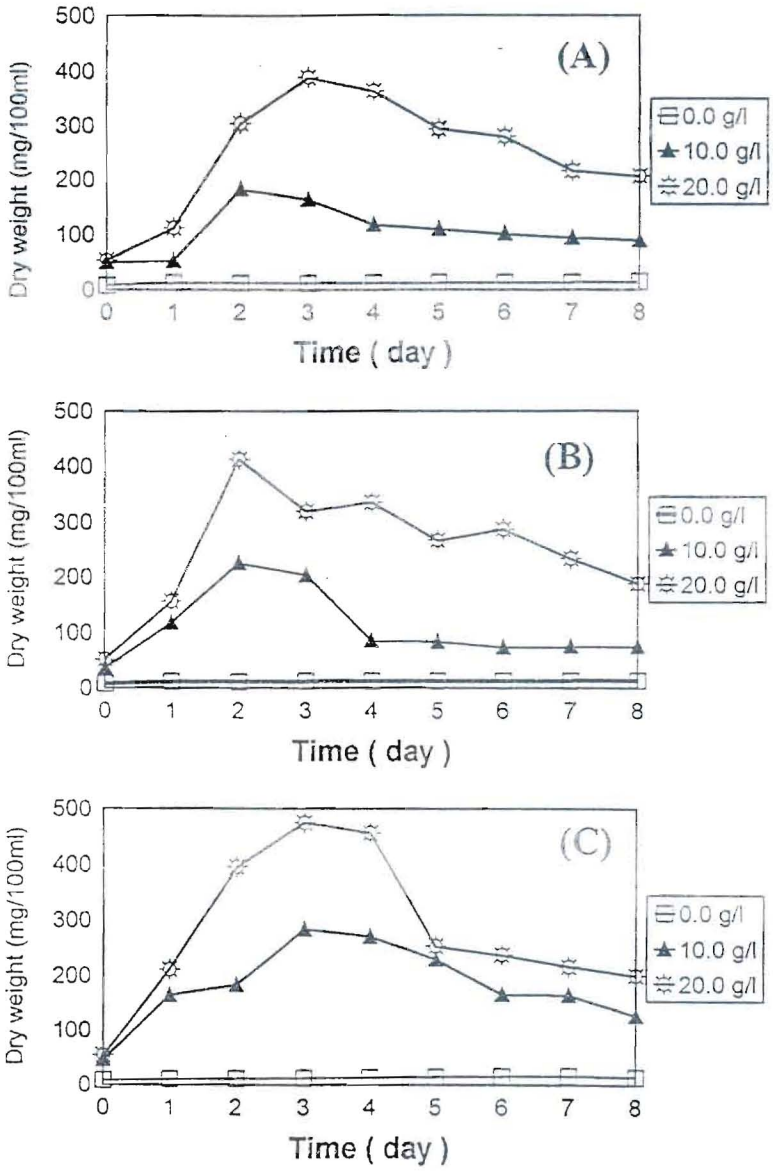

Figure (4) : Effect of different starch concentrations (0.0,10.0 and 20.0 g/l) on the growth of *Pseudomonas stutzeri* (A), *Peanibacillus polymyxa* (B) and a mixed culture of A & B (C).

DISCUSSION

Adhesion of cells to insoluble surfaces is characteristic of a wide variety of biological systems (Bar-shavit, *et al.*, 1977; Imam, *et al.*, 1989) and plays an important role in influencing or regulating cell growth and development as well as other cellular activities (Baier and Weiss, 1975; Kerr *et al.*, 1983).

During manufacture of starch-containing plastics, complexation of starch with polyethylene can affect starch localization within the film and thereby influence its accessibility to an aqueous environment. Imam and Gould (1990) concluded that the type of starch and the way in which it is incorporated into the plastic matrix may influence the ability of amylolytic bacteria to attach to the plastic's surface. This effect is evident in the data presented in (Table 1) since sediment sample (*P. stutzrei)* bacterial cells preferentially adhered to 5% rice or 4% potato starch-polyethylene compared with corn starch-polyethylene regardless of the concentration .While the compost sample (*Peanibacillus polymyxa)* showed a great tendency to adhere to 4% potato starch-polyethylene more than any other tested starch. Both types of bacterial cells showed almost no adhesion to the polyethylene plastic films lacking starch which may indicate that the presence of starch is necessary for cell attachment in that case. It also indicates that the cells may have specific binding sites on their surface to attach them to the starch-plastic films.

Similarly, other investigators remarked that, the successful adhesion of bacterial cells to the starch granules surface was not always parallel to more amylolytic activity of enzymes involved in the degradation process (Teramoto, *et al.*, 1989).

Imam and Gould (1990) observed that the presence of more adhesion sites for bacterial attachment in certain starch-polyethylene formulation doesn't mean more sites sensitive to enzymatic attack in this starch-polyethylene. This is in agreement with previous results obtained by (El-Naggar and El-Aassar, 2000) were *P. stutzeri* and *P. polymyxa* showed a highly significant effect ($p<0.01$) in reducing the elongation percent and tensile strength of 3% potato and 3%corn starch-polyethylene plastic films, respectively, while in the present work the highest adhesion values of these bacteria were 225 and 255 µg/cm2 in case of 4% potato and 5% rice starch-polyethylene plastic films, respectively. So it may be concluded that, the adhesion of *P stutzeri* and *P. polymyxa* to

starch-polyethylene plastic films is not a prerequisite for enzymatic degradation of these films and it may be probably fulfilled by extracellular amylase activity secreted by these strains in the environment or mediated by other different factors and it may be useful to use such bacteria for the degradation of starch plastic films or as alternative sources for the amylase production.

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