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Effect of Carbon and Nitrogen Sources of the Medium used to Grow Biocontrol Strain UTPF61 of *Pseudomonas fluorescens* on its Protective Activity Against Sclerotinia Wilt of Sunflower and its Survival During and after the Formulation Process

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Abstract: Background: Sclerotinia wilt caused by *Sclerotinia sclerotiorum*, is a devastating disease of sunflower worldwide. One promising area of disease management for resource-poor farmers that emerged in recent years in developing countries is the potential of biological control. **Material and methods:** *Pseudomonas fluorescens* strain UTPF61 is an effective biocontrol agent for sclerotinia wilt disease. One major constraint in large-scale field evaluation of biocontrol agents of sclerotinia wilt is their availability in large quantities. Fermentation is important in order to simplify production of biocontrol agents. But the large scale production of biocontrol agent is expensive because of the high cost of the medium. Then this problem is solved with the use of inexpensive agricultural byproducts such as molasses and corn steep. **Results:** This research investigated the effect of some combinations of carbon and nitrogen sources during fermentation of *Pseudomonas fluorescens* UTPF61 on its growth in flask and fermentor and its subsequent antagonistic activity *in vitro* and protective activity in plant. Its survival during the formulation process and shelf life over a period of 150 days was investigated. Rapid growth in flask was observed in medium containing Molasses+yeast extract but medium containing Molasses+2-ammonium hydrogen phosphate had the best effect on the antagonistic efficacy, biocontrol activity, PGPR and survival of bacteria.

Key words: Antagonistic efficiency, antifungal activity, biological control, carbon and nitrogen sources, fermentation, formulation, fluorescent pseudomonads, *Sclerotinia sclerotiorum*

INTRODUCTION

Sclerotinia sclerotiorum is a plant pathogenic fungus that causes destructive diseases of numerous succulent plants (Agrios, 2005). Sunflower (*Helianthus annus* L.) is vulnerable to some pathogens especially the fungus *S. sclerotiorum*.

Biological control is an important component of integrated pest management (Zhang *et al*., 2005). Work on biological control of sclerotinia wilt has intensified (Duffy and Defago, 1999), because no progress in breeding for resistance to sclerotinia wilt in sunflower has been obtained and no effective chemical control of this disease is known (Expert and Digat, 1995). Some strains of *P. fluorescens* were reported to be biocontrol agents of various soil born fungi (Weller, 1988; Defago *et al*., 1990; Expert and Digat, 1995). In spite of these advantages there is little published on commercial production of these agents.

For commercial application, an economical, large-scale mass production method must be developed for *P. fluorescens* and other biocontrol agents. Commercialization of Pseudomonas inocula requires the development of appropriate liquid-culture technology for mass production (Slininger and Sheawilbur, 1995). In this kind of fermentation our goal is to obtain a large amount of microbial agent with high efficacy, high resistance to the drying process involved in formulation and a long

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shelf life. There are some reports that medium components (carbon and nitrogen sources and their ratios, mineral factors, etc.) and conditions (pH, temperature and oxygen transfer) used to grow biocontrol agents, influence their subsequent properties. Fermentation media for production of biocontrol agent should consist of substrates necessary for the growth of microorganism, primarily the carbon, nitrogen and phosphorus sources (Ali *et al*., 2002). Use of commercial products or by-products from food industries, such as molasses, corn steep liquor, malt extract, yeast extract and etc has tended to meet most of the above criteria for production media because they are cheap substrates (Costa *et al*., 2001). Molasses was employed as the basal fermentation media in the stirred fermentor under the submerged fermentation conditions (Ali *et al*., 2002). The importance of inexpensive and readily available media components for commercial products has long been recognizing (Adams *et al*., 2002).

The aims of this study were to find the nitrogen and carbon sources that provide maximum biomass production of *Pseudomonas fluorescens* strain UTPF61, improving the biocontrol potential against the *Sclerotinia sclerotiorum* and its viability during the formulation process.

MATERIALS AND METHODS

Biocontrol strain: *P. fluorescens* UTPF 61 was selected out of 40 strains of fluorescent pseudomonads on the basis of dual culture assays, growth promotion activity on sunflower and production of HCN and protease (Heidary, 2008; Moeinzadeh *et al*., 2010). This strain was originally isolated from the rhizosphere of rice. Stock cultures were prepared for storage at -80°C in 1.5 mL vials by mixing equal volumes of 50% glycerol and 24 h culture broth (from single colony inoculum, 25 mL Luria Bertani medium, 100 mL flask, 130 rpm).

Flask culture studies: The media used in this study were a semi-defined media (M_1, M_2 and M_3), each medium containing in grams per liter: M_1: K_2HPO_4 (0.98 g L^{-1})+$MgSO_4$ (0.4 g L^{-1})+$CaCO_3$ (0.4 g L^{-1}) +molasses (10 g L^{-1})+corn steep (4 g L^{-1}); M_2: K_2HPO_4 (0.98 g L^{-1}) + $MgSO_4$ (0.4 g L^{-1})+$CaCO_3$ (0.4 g L^{-1}) +starch (10 g L^{-1})+corn steep (4 g L^{-1}), M_3: K_2HPO_4 (0.98 g L^{-1})+$MgSO_4$ (0.4 g L^{-1})+$CaCO_3$ (0.4 g L^{-1})+malt extract (10 g L^{-1})+corn steep (4 g L^{-1}). Then in order to investigate the effect of different additional nitrogen sources, different nitrogen sources (tested at 2 g L^{-1}) including 2-ammonium hydrogen phosphate, ammonium sulfate and peptone added to M_1, M_2 and M_3 media separately. Also urea and yeast extract as nitrogen sources and malt extract just added to M_1 medium separately (2 g L^{-1}). This medium was autoclaved for 15 min at 121°C. Flask cultures were inoculated using 1 mL of a 24 h NA culture of UTPF 61 adjusted to 10^6 CFU mL^{-1} in sterile distilled water. Cultures were incubated for 48 h at 120 rpm and 25°C in a rotary shaker incubator, thereafter cell density was determined by cell density standard curve (at 600 nm) and cultures were used for formulation, dual culture assays and greenhouse studies.

Fermentor studies: A fermentor experiment was designed to mimic the industrial fermentation process in which typically there is stepwise scale-up of batch size (Lam *et al*., 1994; Schroth *et al*., 1984). For preparation of seed culture, 50 mL of TSB (Tryptic Soy Broth) medium were inoculated with 1 mL of fresh culture of bacterial suspension with 10^6 CFU mL^{-1}. Then medium incubated for 24 h in a rotary shaker at 26°C and 120 rpm. One milliliter of this culture was transferred to 100 mL of fresh medium in a 250 mL Erlenmeyer flask as a seed culture. This medium was incubated at 25°C under agitation (120 rpm). Finally after 6 h this broth with approximately 10^6 cells mL^{-1} in log phase was transferred to a fermentor under sterile conditions. Before transfer optical density of seed culture were estimated directly by spectrophotometer at 600 nm (PG instruments T70+, England). A 7.5 liter bench-top experimental fermentor (BioFlo 110, New Brunswick Scientific, USA) with a 2 l working volume was operated in batch culture mode. Temperature was kept at 26°C and the initial pH was adjusted to 7. The pH was monitored by a pH probe (Metter Toledo 405-DPAS-SC-k85/325) and Dissolved Oxygen (DO) with a polarographic DO electrode (Ingold Inpro 6800 series), The DO probe was calibrated at 0%, (obtained by briefly disconnecting the cable) and at 100%, (obtained using 800 rpm agitation and 5 l min^{-1} [1 vvm] airflow). An agitation cascade was selected in the controller to maintain DO at 25-30% saturation via automatic adjustment of the agitation speed. To avoid formation of foam, castor oil was added in automatically mode. The volume of seed cultures were 2% (v/v) of fermentor medium. Cells of *P. fluorescens* UTPF61 in different stages of growth (15 and 25 h after inoculation) were harvested in 40 mL aliquots from the fermentor and each aliquot was used for dual culture assays and greenhouse studies. All fermentor experiments were conducted in triplicate.

***In vitro* antagonistic activity:** The *in vitro* inhibition of mycelial growth of *S. sclerotiorum* by the strain UTPF61 was tested using the dual culture technique as described by Keel *et al*. (1996). Three 10 µL drops from different cultivation runs were equidistantly placed on the

margins of Potato Dextrose Agar (PDA) plates and incubated at 25°C for 24 h. Thereafter a 7 mm agar disc from fresh PDA cultures of *S. sclerotiorum* was placed at the centre of the PDA plate and incubated at 20°C for five days. Inhibition of fungal growth was assessed 5 days later by measuring the size of the inhibition zone (in mm).

Formulation and air-drying: Medium containing 9×10^8 CFU mL^{-1} was used for the preparation of a talc-based formulation. To 400 mL of bacterial suspension, 1 kg of purified talc powder (sterilized at 120°C for 15 h), calcium carbonate 15 g (to adjust the pH to neutral) and carboxymethyl cellulose (CMC) 10 g (adhesive) were mixed under sterile conditions, as described by Vidhyasekaran and Muthamilan (1995). The product was air dried at 15°C under sterile conditions until moisture content reached 15-20%. After a further 12 h this product was powdered in a grinder for 1 min and maintained at two temperatures 25 and 4°C in vials. One gram of this formulation was used for viability assays by serial dilution method at determined intervals.

Greenhouse studies: Bacteria that harvested from different cultivation media were tested for their protective activity against *S. sclerotiorum* and their ability of plant growth promotion. Experiments were performed in plastic pots (diameter, 7.5 cm; depth, 8) containing sterilized soil (sand 3, clay 1, humus 1). Surface-sterilized (5% sodium hypochlorite for 3 min and rinsed five times with sterile distilled water) and post germinated seeds of sunflower bacterized by soaking in bacterial suspension (1×10^8 CFU mL^{-1}) for 30 min with a moderate shaking. Bacterial suspensions prepared by resuspending the pelleted bacteria (by centrifuging at 5000 xg for 10 min) from different media by 1% Carboxymethyl cellulose (CMC) slution. Control seeds were shaken only in 1% CMC solution for half an hour. To prepare inoculum of *S. sclerotiorum* for greenhouse studies (protective activity test), millet seeds were sterilized (121°C, 20 min, twice), inoculated with mycelial plugs and incubated at 20°C for 20 days. For protective activity test, 1 g of prepared inoculum was mixed into the upper part of sterile soil, prior to seed planting. For the control treatments, sterile millet seed was mixed into the soil at the same rate. For PGPR test bacterized seeds (as described above) were planted in sterile perlite and irrigated by nutrient solution. Plants were grown in the greenhouse under day and night temperatures of 25/18°C with a 14 h photoperiod and 75% relative humidity. Pots were fully randomized in different places and treatments were as follows: uninoculated seeds (without bacteria = control treatment) with or without fungus and inoculated seed (with bacteria) with or without fungus. Treatments were replicated three times. In antagonistic tests, 30 days after planting, the percentage of healthy plants was determined according to the procedure described by Expert and Digat (1995) and in PGPR test, the length of root and stem, the wet and dry weight of root and stem was calculated after 30 days after sowing.

Statistical analysis: Data were analyzed for significance by analysis of variance, followed by Duncan's multiple range test ($p<0.05$), with the SAS general linear model procedure (SAS Institute, Cary, NC). Normal distribution and homogeneity of variances were checked beforehand. Complete Randomized Design (CRD) was used in all experiments and each treatment was replicated three times.

RESULTS

Effect of different carbon and nitrogen source on growth of UTPF61: Bacteria originating from flask culture in medium containing M_1+yeast extract were more effective on growth of UTPF61 as its optical density was 1.74 after 48 h. Moreover M_1+peptone and M_1+2-ammonium hydrogen phosphate media had a good effect on bacterial growth (Table 1). Bacteria originating from fermentor in medium containing M_1+2-ammonium hydrogen phosphate were more effective on growth of cells as its OD was 2.89 at the end of the cultivation. The effect of other media including M_1+urea and M_1+peptone on growth of bacterium was not significantly different in both of the times (Table 2).

Table 1: Effect of some carbon and nitrogen sources in flask culture on the rate of growth and antagonistic efficacy of *P. fluorescens* strain UTPF61 in 25°C and after 48 h

Media	Log_{10} Cells (mL^{-1})	Inhibition zone(mm)[a]
M_1	13.266^{abcd**}	6.67^{e}
M_1+peptone	14.592^{ab}	12.33^{a}
M_1+2-ammonium hydrogen phosphate	13.812^{abc}	12.33^{a}
M_1+ammonium sulphate	13.032^{abcde}	6.33^{ef}
M_1+urea	12.954^{bcde}	9.67^{c}
M_1+yeast extract	15.372^{a}	6.67^{e}
M_1+malt extract	11.784^{cdef}	0.11^{b}
M_2	11.706^{cdef}	6.33^{ef}
M_2+peptone	11.55^{cdef}	9.67^{c}
M_2+2-ammonium hydrogen phosphate	11.16^{def}	8.17^{d}
M_2+ammonium sulphate	12.408^{bcde}	8.33^{d}
M_3	10.614^{ef}	5.5^{fgh}
M_3+peptone	10.848^{def}	5.33^{gh}
M_3+2-ammonium hydrogen phosphate	9.834^{f}	0.5^{h}
M_3+ammonium sulphate	11.16^{def}	0.6^{efg}

[a]Width of the zone of inhibition was measured from the edge of the fungal colony to the edge of the bacterial colony, statistical significance was judged at the $p<0.01$, **OD600

Table 2: Effect of some culture media on the rate of growth and antagonistic efficacy of *P. fluorescens* strain UTPF61 grown in fermentor in 26°C and with two different times 15 and 25 h after incubation time

Media	Log_{10} cells (mL^{-1})	Inhibition zone(mm)[a]
M_1+peptone (after 15 h)	17.4312[a]**	12.67[bc]
M_1+peptone (after 25 h)	20.442[b]	11b[c]
M_1+2-ammonium hydrogen phosphate (after 15 h)	24.342[a]	12.3[a]
M_1++2-ammonium hydrogen phosphate (after 25 h)	24.108[a]	12.3[a]
M_1+urea (after 15 h)	21.144[b]	10.33[c]
M_1+urea (after 25 h)	19.74[b]	10.33[c]

[a]Width of the zone of inhibition was measured from the edge of the fungal colony to the edge of the bacterial colony, statistical significance was judged at the p<0.01, **OD600

***In vitro* antagonistic test:** Bacteria originating from flask culture in media containing M_1+peptone or M_1+2-ammonium hydrogen phosphate showed protective activity (12.33 mm inhibition zone on the medium used) against *S. sclerotiorum* (Table 1). Bacteria originating from fermentor in medium containing M_1+2-ammonium hydrogen phosphate, showed protective activity (12.33 mm inhibition zone on the medium used) against *S. sclerotiorum* in both of the times 15 and 25 h after inoculation and the effect of this medium on the protective activity of UTPF61 was significantly more than those of other media (Table 2).

Greenhouse experiment: Media containing M_1+2-ammonium hydrogen phosphate or M_2+2-ammonium hydrogen phosphate showed a strong inhibition against *S. scletoriorum*, yielding 84.47% healthy plants after 30 days (Fig. 1). Investigation of plant growth promotion test in greenhouse trials showed that cells of UTPF61 grown in M_1+2-ammonium hydrogen phosphate were more effective in increasing the root and stems length and dry and wet weight of plants. Also its effect was significantly different with control treatment. Length of roots in all of the treatments was same but they were significantly different with control treatment (Table 3).

Bacteria originating from fermentor in media containing M_1+2-ammonium hydrogen phosphate and M_1+peptone at 15 h after inoculation, showed more antifungal activity than those of the other media (Fig. 2). Biocontrol activity of cells that harvested at 15 h was better than those of 25 h. Moreover, bacteria obtained from M_1+2-ammonium hydrogen phosphate were more effective in increasing the length of roots in both of times but bacteria obtained from M_1+2-ammonium hydrogen phosphate at the time of 25 h improved its effect on the growth factors (Table 4). Bacteria obtained from M_1+urea were like bacteria obtained from M_1+2-ammonium hydrogen phosphate that bacteria in the second time

Table 3: Effect of different carbon and nitrogen sources in flask culture on ability of bacteria to improved the growth factors of sunflower

Media	Dry weight	Wet weight	Length of stem	Length of root
M_1	0.24[c]	2.68[c]	19.33[bc]	18.33[a]
M_1+2-ammonium hydrogen phosphate	0.32[a]	3.93[a]	24.33[a]	16.67[a]
M_1+peptone	0.27[bc]	3.12[bc]	21.67[ab]	16.67[a]
M_1+urea	0.3[ab]	3.7[ab]	42.17[ab]	17[a]
M_2	0.17[d]	2.67[c]	16[cd]	16.67[a]
M_3	0.23[c]	3.2[bc]	17[cd]	16[a]
control	0.13[d]	1.48[d]	13.67[d]	11[b]

statistical significance was judged at the p<0.01

Table 4: Effect of Bacteria obtained from fermentor medium containing: M_1+2-ammonium hydrogen phosphate on growth factors of sunflower in two times 15 and 25 h after inoculation

Bacteria obtained from M_1+2-ammonium hydrogen phosphate in two times after inoculation	Dry weight	Wet weight	Length of stem	Length of root
After 15 h	0.36[b]	4.2[a]	24[c]	18[a]
After 15 h (control)	0.44[a]	4.06[ab]	26.67[b]	9.33[c]
After 25 h	0.41[ab]	4.09[ab]	28.17[a]	15.67[b]
After 25 h (control)	0.28[c]	3.31[b]	28.67[a]	8.67[c]

Statistical significance was judged at the p<0.01

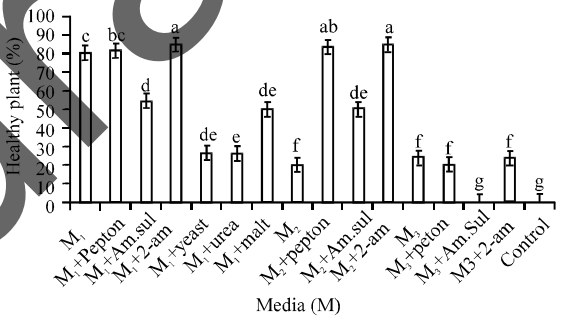

Fig. 1: Biocontrol activity of *Pseudomonas fluorescens* UTPF61 grown in flask media with different combinations of nitrogen and carbon sources, against *Sclerotinia sclerotiorum* under greenhouse conditions. Am.sul: Ammonium sulphate, 2-am: 2-ammonium hydrogen phosphate. Percent of healthy plants determined after 30 days. Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test

after incubation were more effective than the first time (Table 5) but about bacteria obtained from M_1+peptone harvested bacteria in the time of 15 h were more effective than the time of 25 h (Table 6).

Formulation and air-drying: Shelf-life of formulated bacteria obtained from different media was investigated in two temperatures. In all of the treatments, population of UTPF61 decreased in both of temperatures (4 and 25°C), but the level of reduction was not the same for all

treatments. The population of cells from M_3+peptone had higher decline whereas the sensitivity of cells from M_1+2-ammonium hydrogen phosphate to air drying was lower than those of other treatments. Almost in all treatments UTPF61 survived up to 90 days and in some of them up to 150 days without any dramatic decline from the initial population (Fig. 3, 4).

Table 5: Effect of Bacteria obtained from fermentor medium containing: M_1+peptone on growth factors of sunflower in two times 15 and 25 h after inoculation

Bacteria obtained from M_1+2-ammonium hydrogen phosphate in two times after inoculation	Dry weight	Wet weight	Length of stem	Length of root
After 15 h	0.34^{a}	4.07^{a}	26^{a}	14.33^{a}
After 15 h (control)	0.25^{b}	3.33^{b}	25^{ab}	10.33^{bc}
After 25 h	0.25^{b}	3.04^{b}	23^{b}	11.67^{bc}
After 25 h (control)	0.25^{b}	3.18^{b}	24.17^{ab}	12.33^{c}

Statistical significance was judged at the $p<0.01$

Table 6: Effect of Bacteria obtained from fermentor medium containing: M_1+urea on growth factors of sunflower in two times 15 and 25 h after inoculation

Bacteria obtained from M_1+2-ammonium hydrogen phosphate in two times after inoculation	Dry weight	Wet weight	Length of stem	Length of root
After 15 h	0.32^{ab}	3.51^{a}	23.33^{ab}	12.67^{b}
After 15 h (control)	0.27^{b}	2.87^{b}	23^{ab}	11.33^{b}
After 25 h	0.35^{a}	3.71^{a}	23.33^{a}	14.67^{a}
After 25 h (control)	0.3^{ab}	3.34^{a}	21.67^{b}	12.33^{b}

Statistical significance was judged at the $p<0.01$

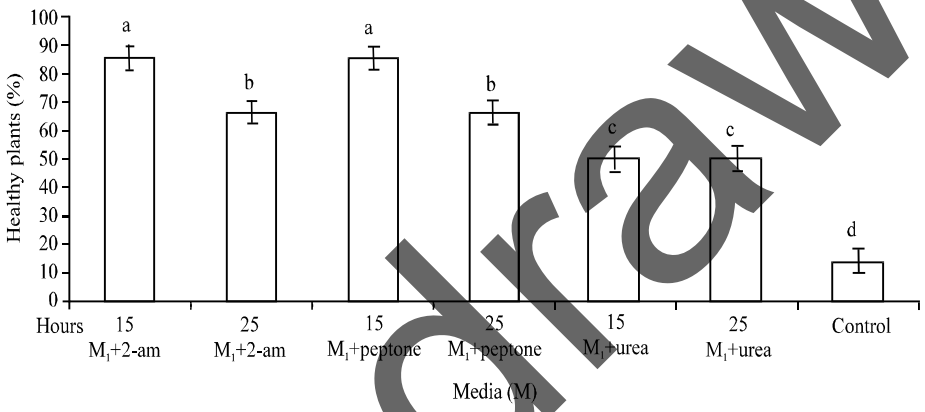

Fig. 2: Biocontrol activity of *Pseudomonas fluorescens* UTPF61 against *Sclerotinia sclerotiorum* grown in a fermentor with different of nitrogen sources, against *Sclerotinia sclerotiorum* under greenhouse conditions. 15 h: 15 h after inoculation of bacteria to fermentor's media, 25 h: 25 h after inoculation of bacteria to fermentor's media. Percent of healthy plants determined after 30 days. Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test, Am.sul: Ammonium sulphate, 2-am: 2-ammonium hydrogen phosphate

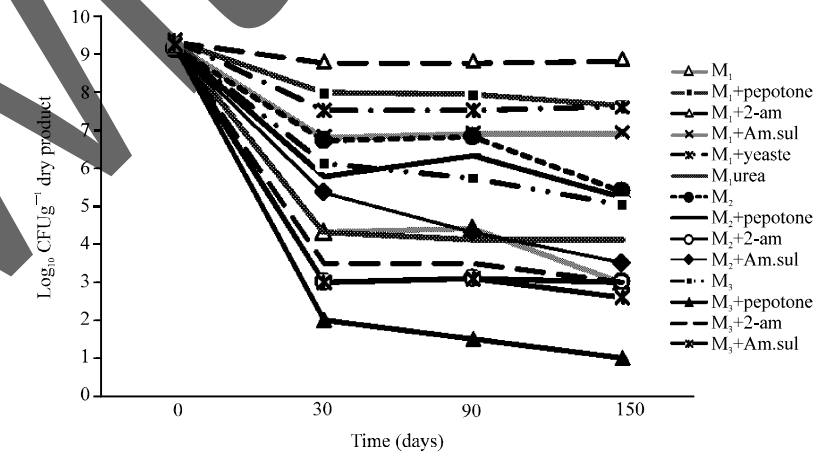

Fig. 3: Survival of *Pseudomonas fluorescens* UTPF61 grown in culture media with different carbon and nitrogen sources during air drying and maintenance of its talc-based formulation for 150 days in vials at 25°C, Am.sul: Ammonium sulphate, 2-am: 2-ammonium hydrogen phosphate

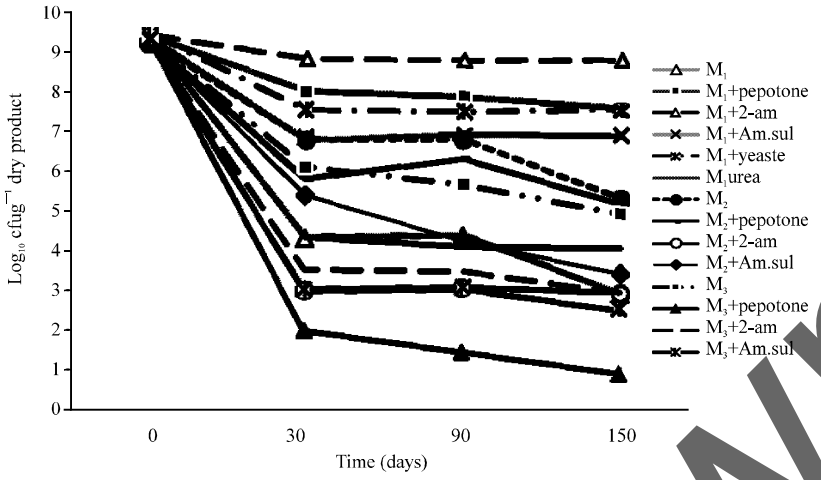

Fig. 4: Survival of *Pseudomonas fluorescens* UTPF61 grown in media with different carbon and nitrogen sources during air drying and maintenance of its talc-based formulation for 150 days in vials at 4°C, Am.sul: Ammonium sulphate, 2-am: 2-ammonium hydrogen phosphate

DISCUSSION

Since the commercial production of *P. fluorescens* for application as a biocontrol agent requires both low cost and high cell density, so the finding the best nutritional supplements and environmental conditions is important for mass production of this agent.

The results obtained in this study showed that the nature of carbon and nitrogen sources in culture media influences the rate of bacterial growth but notably the medium showing more effect on the bacterial growth, had not more effect on protective activity, in other word, no correlation was found between the rate of bacterial growth and antagonistic efficacy. This is in common with the findings by Duffy and Defago (2000) and Costa *et al.* (2001).

Moreover, according to the flask culture studies, maximum of growth observed in M_1+2-ammunium hydrogen phosphate and there wasn`t significant difference between two harvesting times. But the amazing results happened about M_1+urea and M_1+peptone that unlike to the previous experiments in flask there wasn't any significant difference in both of the times between these two media from the rate of growth. In general in this study in one ml of cultivation media the concentration of bacterium is more than one ml of media in the experiments on flasks.

There are reports that medium and conditions used for production of biocontrol agents, influence their ability to survive during the formulation process (Slininger *et al.*, 1996; Zhang *et al.*, 2005). For example mild thermal and pH stresses and carbon starvation can increase the resistance of cells to further stresses (Van Overbeek *et al.*, 1995; Givskov *et al.*, 1994a, b; Jenkins *et al.*, 1988; Oestling *et al.*, 1993). There are different hypotheses to explain these phenomena. In most of these studies resistance of cells was the result of expression of shock-inducible proteins like heat and cold shock proteins. In our study bacterial populations that were grown in medium with a combination of M_1+2-ammonium hydrogen phosphate has a lowest decrease after formulation. Perhaps this combination of carbon and nitrogen stimulated expression of stress-tolerance proteins.

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