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INTERGENERIC MICROBIAL CO-FLOCS: POSITIVE ROLE OF BIOTIC AND ABIOTIC FACTORS ON THE ENHANCEMENT OF CO-FLOCCULATION BETWEEN PGPR CELLS

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Abstract :

The positive effect of certain biotic and abiotic factors viz., Inoculum level and growth phase, cultural condition, pH, presence of divalent cations and chelating agents on the enhancement of co-flocculation between PGPR cells viz., Pseudomonas fluorescens (PF-5) and Paenibacillus polymyxa (PB-5) was studied under in vitro condition.

It was observed that 107:107 inoculum level and stationary growth phase of PGPR partners were found to be optimum for maximising the coflocculation. The cells of PGPR partners harvested from N-deficient medium recorded more co-flocculation when compared to PGPR cells harvested from N-rich medium. Further, The PGPR cells cultured at 350c and at pH level of 7.5 recorded higher co-flocculation percentage than other levels. Moreover, the addition of Ca2+, as divalent cations, augmented higher co-flocculation percentage of PGPR cells while the addition of EDTA, as chelating agent, reduced the stability of PGPR coflocs, significantly.

It was concluded that the PGPR cells cultured in N-deficient medium at 350C temperature and pH 7.0 levels during the stationary growth phase yielded more coflocculation percentage between PGPR partners. Moreover, the use of 107::107 inoculum level of PGPR partners and addition of calcium, as divalent cation, were found to be optimum for maximising the coflocculation percentage between PGPR partners.

KEYWORDS :

PGPR, biotic and abiotic factors, coflocculation, divalent cations, chelating agents.

INTRODUCTION:

Freeliving, beneficial, rhizobacteriae have been shown to improve the plant growth and yield and are usually referred as "plant growth promoting rhizobacteria" (PGPR) or by one group of workers in china as "yield increasing bacteria" (YIB) (Tang, 1994). PGPR may increase plant growth by different mechanisms viz., nitrogen fixation (Hong *et al*., 1999), regulating ethylene production in roots (Glick, 1995) releasing phytohormones (Frankenberger and Arshad, 1995), synthesizing siderophores (Budzikiewicz,1997) and indirectly either by the suppression of well known diseases caused by major pathogens or by reducing the deleterious effect of minor pathogens (Shishido and Chanway, 1999). PGPR include bacteria belonging to the genera, namely, *Pseudomonas* and *Paenibacillus*. The beneficial effects of *Pseudomonas s* and *Paenibacillus,* as PGPR, have been reported by many authors (Suslow and Schroth, 1982; Glandorf *et al*., 1994 Hofte *et al.*, 1991; Timmusk, 2003; Yao *et al*.,2006; Raza et al., 2008) and the same are used as agricultural bioinoculant, worldwide, for the enhancement of growth and yield of many important field crops, including, lowland rice. However, the use of the agricultural bioinoculant recorded a poor performance in natural environment and in the rhizosphere of host plants.vanVeen et al. (1997) critically reviewed the reasons for the same and suggested that instead of trying single microbial strain with single trait, as agricultural bioinoculant, trying to use microbial consortia for harnessing multiple benefits.

Higher degree of stress tolerance, longer shelf life, enhanced survivability in soils and on seeds and consistent plant response to inoculation are the important characteristics of any agricultural bioinoculant (Neyra *et al.*, 1997). Okon and Labaendra-Gonzalez (1994) suggested the importance of the physiological status of the microorganisms in agricultural bioinoculant rather than their cell numbers to ensure more survival in carrier materials, survival in soil and on seed, colonisation in the rhizosphere and positive plant response to bioinoculation. Some of the most promising research topics on

novel agricultural bioinoculant technology, include, the use of flocculated cell forms of microorganisms, as delivery system (Olubayi *et al.,* 1998). Cell aggregation/flocculation is a widespread phenomenon in the microbial world and occurring under certain physiological conditions (Callega, 1984). Neyra *et al.*(1999) proposed the use of "Intergeneric microbial coflocs", as a novel delivery system and a new generation of agricultural bioinoculant. Further, the microbial coflocs contained high cell titre, increased adhesiveness to plant roots, enriched in encysted cells with thick capsules surrounded by the EPS rich network which provided higher stress tolerance and longer shelf life to bioinocula.

In the present investigation, the positive role of various biotic and abiotic factors viz., Inoculum level, growth phase, culture media, growth temperature, pH, divalent cations and chelating agents on the enhancement coflocculation between the PGPR cells *viz., Pseudomonas* and *Paenibacillus* was studied.

2. MATERIALS AND METHODS

2.1. Preparation of inoculums

The PGPR strains *viz., Pseudomonas fluorescens* (PF-5) and *Paenibacillus polymyxa* (PB-5) were grown in King´s B and Nutrient glucose broth, respectively, maintained in a sshaking bath at 30±2^{0}C for 5 days to get stationary phase cultures. Then, each medium was centrifuged, separately, at 5000×g for 10 min to harvest the stationary phase cells and the pellets washed three times with 0.1 M phosphate buffer (pH 6.8). Finally, the PGPR cells were resuspended, separately, in the same buffer to get a cell concentration of 1 ×107 CFU ml-1 by measuring the absorbance at 420 nm and used as inoculum.

2.2. Preparation of co-aggregation buffer (Grimaudo andNesbitt, 1997)

The co-aggregation buffer was prepared with the following composition: 20 mM Tris- HCI buffer; 0.01 mM CaCl2; 0.15 M NaCl and 0.02% NaN3.

2.3. Co-aggregation assay (Jabra-Rizk et al., 1999)

One ml aliquot of each bacterial culture *viz.,Pseudomonas fluorescens* (PF-5) and *Paenibacillus polymyxa* (PB-5) was mixed together in 10 ml Co-Ag buffer. The mixture was vortexed for 10s, shaken on a rotary platform shaker for 3 min. and left undisturbed for 24h. Uninoculated buffer served as control. All Co-Ag reaction were performed in triplicate.

2.4. Estimation of co-aggregation percentage (Madi and Henis, 1989)

After the incubation period, the aggregates settled at the bottom of the tube while some of the free cells remained in suspension. The supernatant was sampled and the turbidity measured in spectronic-20 colorimeter at 420nm. Then the flocs were mechanically dispersed by treating in a tissue homogenizer for 1 min and the total OD was measured and the percent of coaggregation was calculated as follows:

$$\%\text{Co} - \text{aggregation} = \frac{(OD_t - OD_s)\ x1OO}{OD_s}$$

Where,

ODt = total optical density after mechanical dispersion and

ODs = OD of supernatant after aggregate had settled

2.5. Factors affecting the coaggregation of PGPR cells

2.6. Effect of cell age on co-aggregation of PGPR isolates

The PGPR isolates *viz., Pseudomonas fluorescens* (PF-5) and *Paenibacillus polymyxa* (PB-5) were grown in King's B and Nutrient glucose broth, respectively, in a shaking broth at 30 ± 2°C.The lag, log and stationary phase cultures of the PGPR cells were harvested at 0, 24 and 72 hours, respectively, and the co-aggregation percentage was estimated.

2.7. Effect of temperature on co-aggregation of PGPR cells

The PGPR isolates, namely, PF-5 and PB-5 were grown separately for 5 days as per the conditions mentioned above but the temperature was maintained at different levels, namely 25, 30, 35, 40 and 45°C for the growth of the isolates. After 72 h incubation, the coaggregation percentage of the PGPR isolates, maintained at different levels of temperature, was estimated.

2.8. Effect of pH on co-aggregation of PGPR cells

The PGPR isolates, namely, PF-5 and PB-5 were grown for 72h as per the conditions mentioned above. After 72h of incubation, the cells of each bacterial isolates were harvested and the co-aggregation percentage was estimated according to Madi and Henis (1989) in coaggregation buffer (Grimaudo and Nesbitt, 1997) maintained at different pH levels, namely, 6.0, 6.5, 7.0 and 7.5.

2.9. Effect of culture media on co-aggregation of PGPR cells

The PGPR isolates, namely, PF-5 and PB-5 were grown in King's B and Nutrient glucose broth,respectively, broth under N free and N supplemented condition in a shaking bath at 35ºC for 72h. After the incubation period, the cells of each PGPR cells were harvested separately and the co-aggregation percentage was estimated as stated earlier in co-aggregation buffer (Grimaudo and Nesbitt,1997) maintained at a pH level of 7.5.

2.10. Effect of cell number on co-aggregation of PGPR cells

The PGPR isolates, namely, PF-5 and PB-5 were grown for 72h in King's B broth and Nutrient glucose broth, respectively as per the condition mentioned above. After the incubation period, the cells of each PGPR isolates were harvested separately and the co-aggregation percentage was estimated at different levels of cell number, namely, 104:104, 105:105 and 106: 106, 107: 107and 108:108.

2.11. Effect of divalent cations on co-aggregation of PGPR cells

The PGPR isolates, namely, PF-5 and PB-5 were grown in Kings'B broth, and nutrient glucose broth respectively, at 35 ^{0}C for 72h. Then, the cells were harvested and the co-aggregation percentage was estimated as stated elsewhere in the text in the coaggregation buffer (Grimaudo and Nesbitt, 1997)

supplemented with different divalent cations viz., Ca2+, Mg2+ and Ba2+, with a view to test their efficacy on the induction of co-aggregation, at 0.1mM level.

2.12. Effect of chelating agents on co-aggregation of PGPR cells

The PGPR isolates, namely, PF-5 and PB-5 were grown in N-free Kings'B and Nutrient glucose broth, respectively and the coaggregation percentage was estimated in coaggregation buffer (Grimaudo and Nesbitt, 1997) maintained at pH 7.5 together with the addition of EDTA (Ethylene diamine tetra acetic acid) or EGTA (Ethylene glycol-bis-(□-amino ethyl ether) N-N'tetracetic acid) at 1mM level.

3. RESULT AND DISCUSSION

In the present study, the PGPR genera *viz., Pseudomonas* and *Paenibacillus*, recorded the highest intergeneric coflocculation percentage at a inoculum level of 107:107 and any increasing or decreasing values to this level of inoculum caused a reduction in their coflocculation ability (Fig-1). The importance of inoculum level of coaggregating partners in intergeneric coflocculation has been reported by Gibbons and Nygaard (1970), Kolenbrander and Andersen, (1986) and Kolenbrander, (1988). Gibbons and Nygaard (1970) reported the importance of equal number of coaggregating partners to obtain a visibly stronger coflocculation. The higher inoculum levels leads to the formation of rosette type colonies that are visible only under microscope (Kolenbrander and Andersen, 1982). The results of the present study clearly revealed the importance of inoculum level of coaggregating PGPR partners to achieve the maximum coflocculation percentage. The effect of different growth phases of the coflocculating partner's *viz., Pseudomonas* and *Paenibacillus* on the coflocculation percentage revealed that the growth phase of the coaggregating partners played a critical role on the coflocculation percentage of the same. Among the different growth phases tested, the stationary growth phase of coaggregating PGPR partners recorded the highest coflocculation percentage followed by lag and log growth phases (Fig-2). The lowest coflocculation percentage recorded with the log growth phase of the coaggregating partners and revealed the fact that active metabolic state of the microbial cell was not conducive for coflocculation. Kolenbrander *et al*.(1983) and Kolenbrander and Williams (1983) reported the effect of growth phases on the coflocculation of Streptococcus with other human oral bacterial isolates, collected from the same site. The effect of the culture age on the composition of the cell surface of bacteria has been reported by many workers (Burdman *et al*., 1998; Burdman *et al.*, 2000). Nikitina *et al*. (2000) reported that the coflocculation of Azospirillum brasilense SP7 (S) and SP.7.2.3 changed with the culture age. The results of the present study clearly revealed the determining role of growth phase of PGPR cells on coflocculation percentage of the same. The effect of cultural conditions viz., N-free and N- supplementation conditions of *Pseudomonas* and *Paenibacillus* cells on the coflocculation percentage revealed that the cultural conditions of the coaggregating partners played a key role in determining the coflocculation percentage of PGPR cells. Between the two cultural conditions tested, namely, PGPR cells grown in N-free medium and PGPR cells grown in N supplemented medium, the PGPR cells collected from N-free medium recorded more coflocculation percentage Sadasivan and Neyra (1985) reported the effect of high C:N ratio on the coflocculation of *Azospirillum brasilense* Cd. strains. Kolenbrander, (1988) summarized the effect of culture medium on the coflocculation of Streptococci and Actinomyces suspensions, collected from human oral ecosystem. Burdman *et al*. (1998) reported that when Azospirillum brasilense strains FAJ0204 grown under high C: N ratio medium accumulated high amount of poly-beta-

hydroxybutyrate with a change in cell surface properties, namely, a well-defined electron-dense layer outside the outer membrane. It has been previously reported that the cells of Azospirillum brasilense growing in culture medium at high C: N ratio tends to aggregate and it was also shown that the amount of arabinose present in the EPS correlated with the extent of cell coflocculation of different Azospirillum strains (Burdman *etal.*, 2000). The results of present study also revealed the highest coflocculation percentage of PGPR cells, *viz., Pseudomonas* and *Paenibacillus* collected from N deficient medium which affected the cell surface characteristics of PGPR cells and resulted in more coflocculation percentage of the same. The effect of different growth temperature levels viz., 25, 30, 35, 40 and 45 on the coflocculation percentage of PGPR cells *viz., Pseudomonas* (PF-5) and *Paenibacillus* (PB-5), revealed that the growth temperature level of PGPR cells played a critical role in determining the coflocculation percentage of the PGPR partners. The increasing level of growth temperature showed an increasing trend in coflocculation percentage upto 35°C and thereby a reduction in the same was observed (Fig-4). Kestrup and Funder-Nielsen (1974) Henis(1989),reported the positive effect of growth temperature in determining the coflocculation of Streptococci with Fusobacterium and Actinomyces. Burdman et al. (1998) reported the positive effect of growth temperature on coflocculation of *Azospirillum brasilense* cd. cells. They reported that *Azospirillum* grown under high C: N ratio recorded a higher coflocculation at higher growth temperature levels whereas the highest temperature beyond the optimum growth temperature level caused dispersion of the coaggregates. The effect of different levels of buffer pH on the coflocculation percentage of PGPR cells v*iz., Pseudomonas* (PF-5) and *Paenibacillus* (PB-5) revealed the positive role of buffer pH on the coaggregating PGPR partners to attain maximum coflocculation percentage. Among the different buffer pH levels tested, the 7.5 level of buffer pH, recorded the highest coflocculation percentage of PGPR partners followed by 6.5, 6.0 and 7.0 buffer pH levels (Fig-5). reported the positive effect of pH on coflocculation of *Azosprillum* cells and added that there was dispersion of *Azospirillum* cells at neutral pH (pH 7.0) while any increase or decrease to this pH level augmented the coflocculation of *Azospirillum* cells. Burdman *et al*. (1998) reported the involvement of charged groups in this phenomenon and the Azospirillum strains Cd and FAJ 0204 responded differentially to the levels of pH. They also added that the negative ionized groups of bacterial cell surface could be neutralized by protonation and thus reducing the strength of repulsive forces between the bacteria which lead to coflocculation. The results of the present study also revealed the differential response of PGPR cells to different pH levels for coflocculation. The effect of addition of different divalent cations viz., Ca2+, Mg2+ and Ba2+ to Co-Ag buffer on the coflocculation percentage of PGPR cells *viz., Pseudomonas* (PF-5) and *Paenibacillus* (PB-5) revealed the positive effect of divalent cations in augmenting coflocculation percentage of PGPR cells. Among the different divalent cations tested, the addition of Ca2+ was found to augment the phenomenon to a higher level followed by Mg2+ and Ba2+ (Fig-6). Jana (1998) reported the positive role of Ca2+ ions in the augmentation of cell surface hydrophobicity in *Pseudomonas fluorescens*. Rose (1984) reported the positive role of divalent cations on the flocculating ability of S. cerevisiae. Mill (1964) reported that Ca2+ ions acting as bridges in the coflocculation of yeast cells. Miki et al. (1982) emphasized the importance of Ca2+ ions in yeast cell flocculation but in some cases magnesium and manganese ions may act as substitutes. The results of the present study clearly revealed the importance of Ca2+ ions in augmenting the coflocculation of PGPR cells. The effect of addition of chelating agents viz., EDTA and EGTA to coflocculation buffer on the coflocculation percentage of PGPR cells *viz., Pseudomonas* (PF-5) and *Paenibacillus* (PB-5) revealed the positive role of chelating agents on the reduction of coflocculation. Percentage between PGPR cells. Between the two chelating agents tested, the addition of EDTA to the Co-Ag buffer reduced the coflocculation percentage of PGPR cells to a marked level followed by EGTA (Fig-7). Burdman et al. (1998) reported the effect of EDTA and EGTA on the dispersion of *Azospirillum coaggregates*. They suggested the involvement of outer membrane proteins of microbial cells in cell-to-cell adhesion. They also added that higher concentrations of these compounds drastically reduced the cell viability and caused partial lysis of bacteria. Madi and Henis (1989) showed the treatment with NaEDTA resulted in the dispersion of *Azospirillum* aggregates while the addition of dialyzed EDTA restored their coflocculation capacity and suggested the role surface-located proteins involved in the coflocculation process. The results of the present study also revealed the positive influence of EDTA in reducing the coflocculation of PGPR cells and emphasized the role of surface located protein in coflocculation.

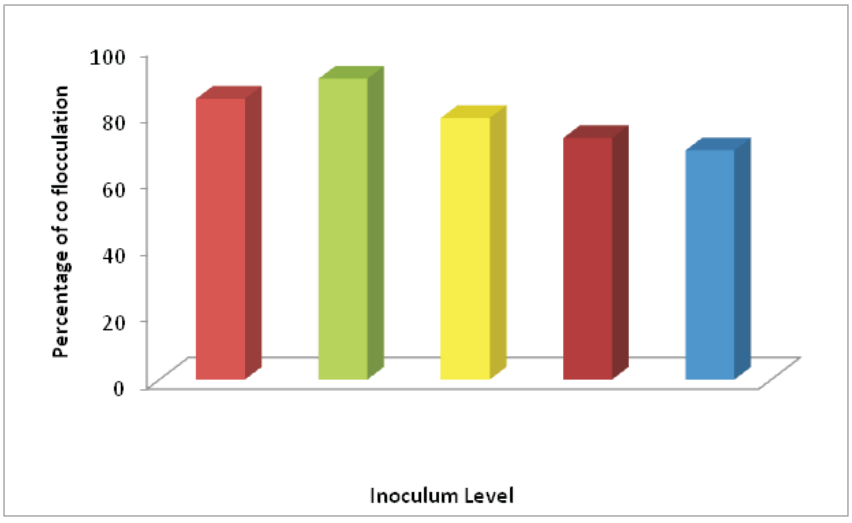

Fig 1 Effect of different inoculum levels on coflocculation of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*

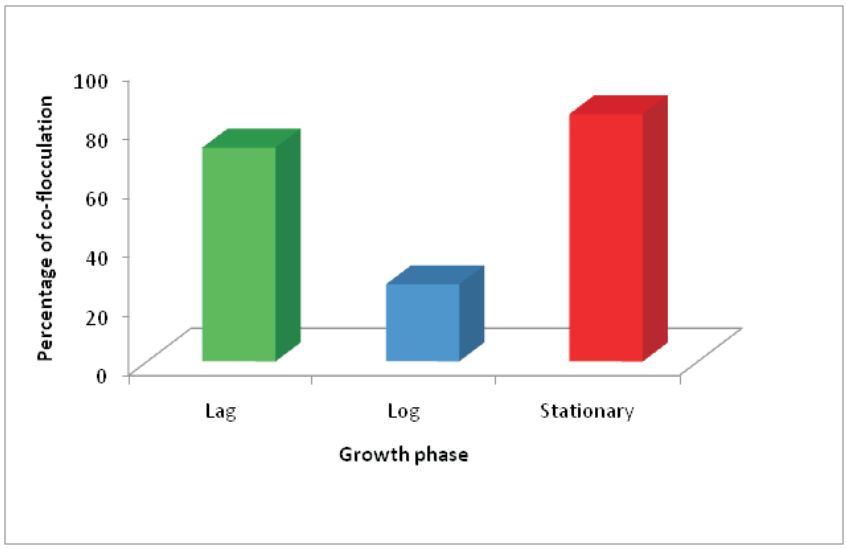

Fig 2 Effect of different growth phases on coflocculation of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*

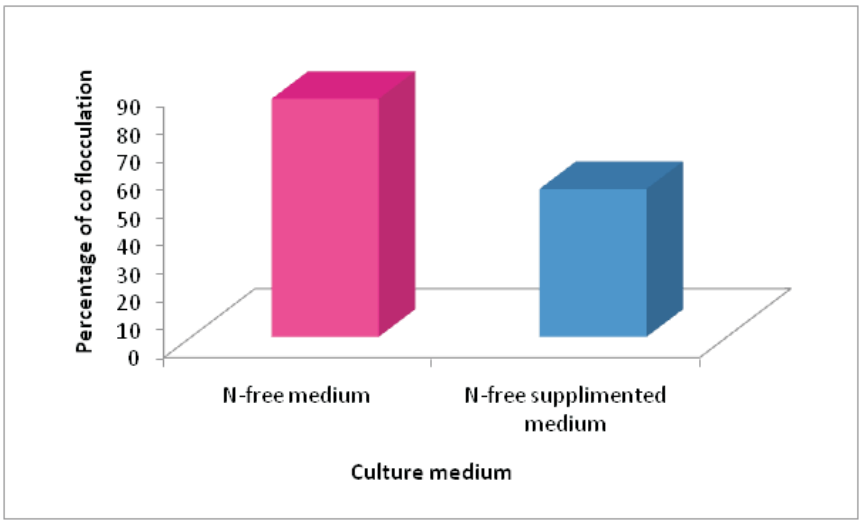

Fig 3 Effect of different culture media on coflocculation of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*

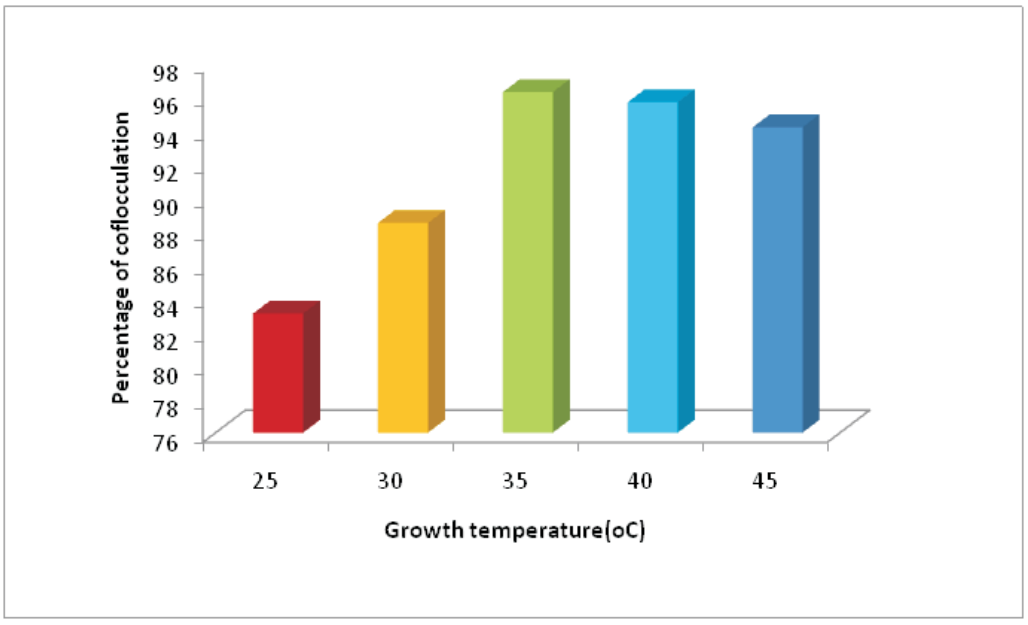

Fig 4 Effect of different growth temperature levels on coflocculation of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*

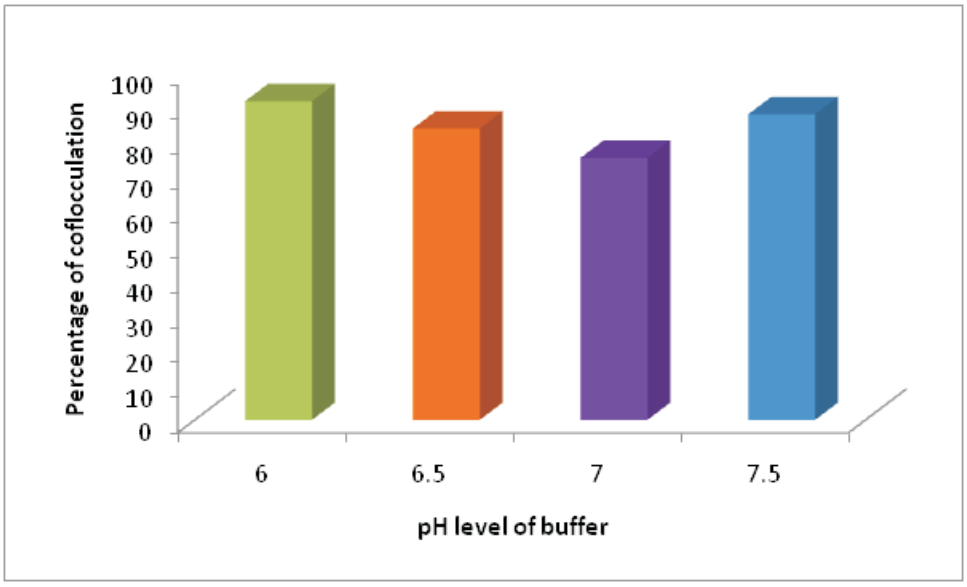

Fig 5 Effect of different level of pH on coflocculation of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*

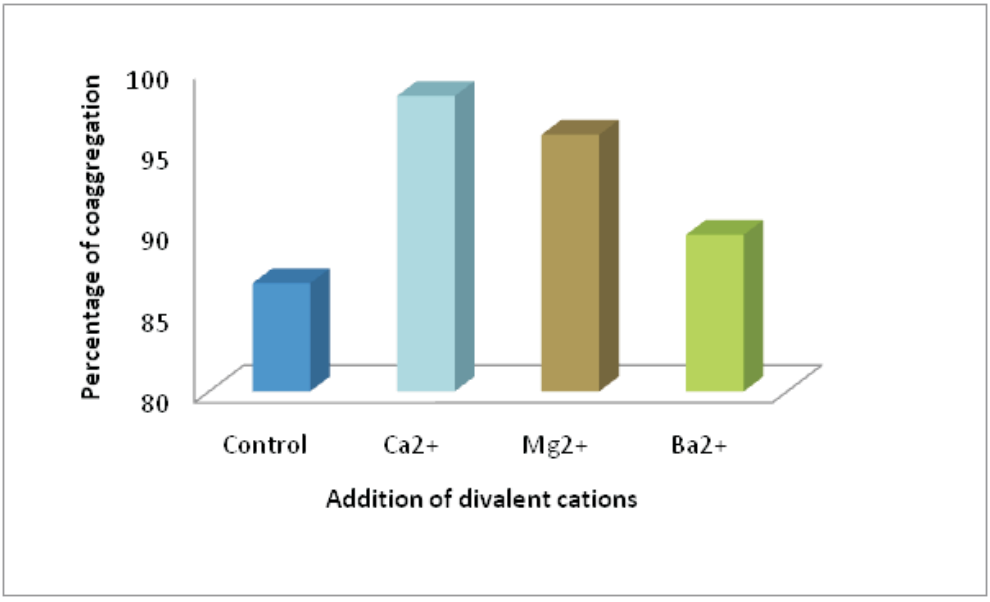

Fig 6 Effect of addition of divalent cations on coaggregation of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*

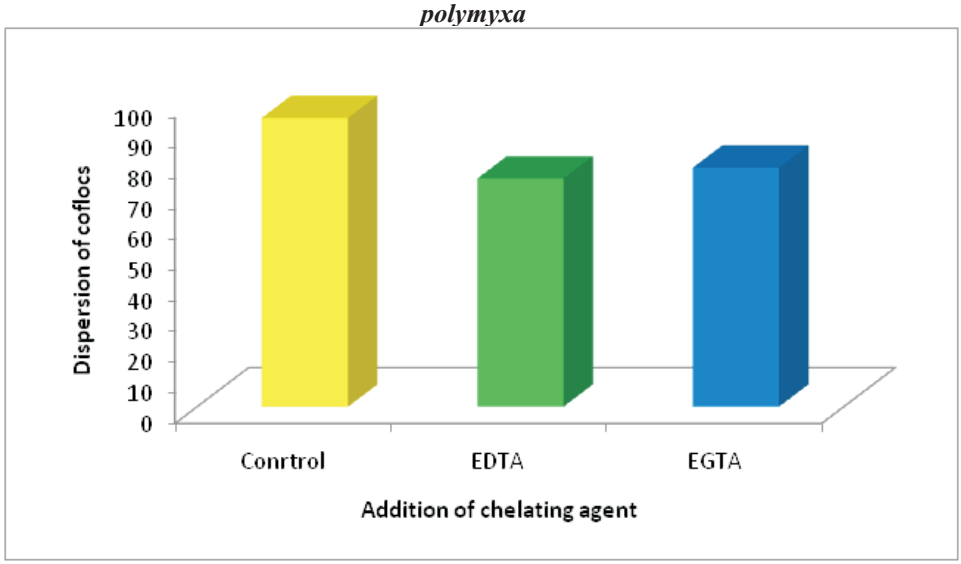

Fig 7 Effect of addition of chelating agent on dispersion of co flocs of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*

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