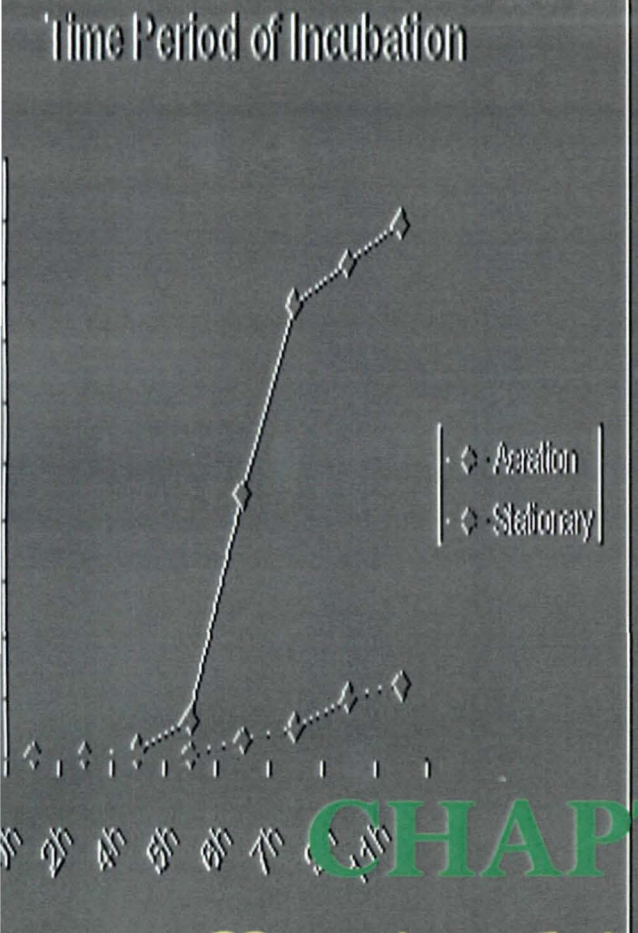
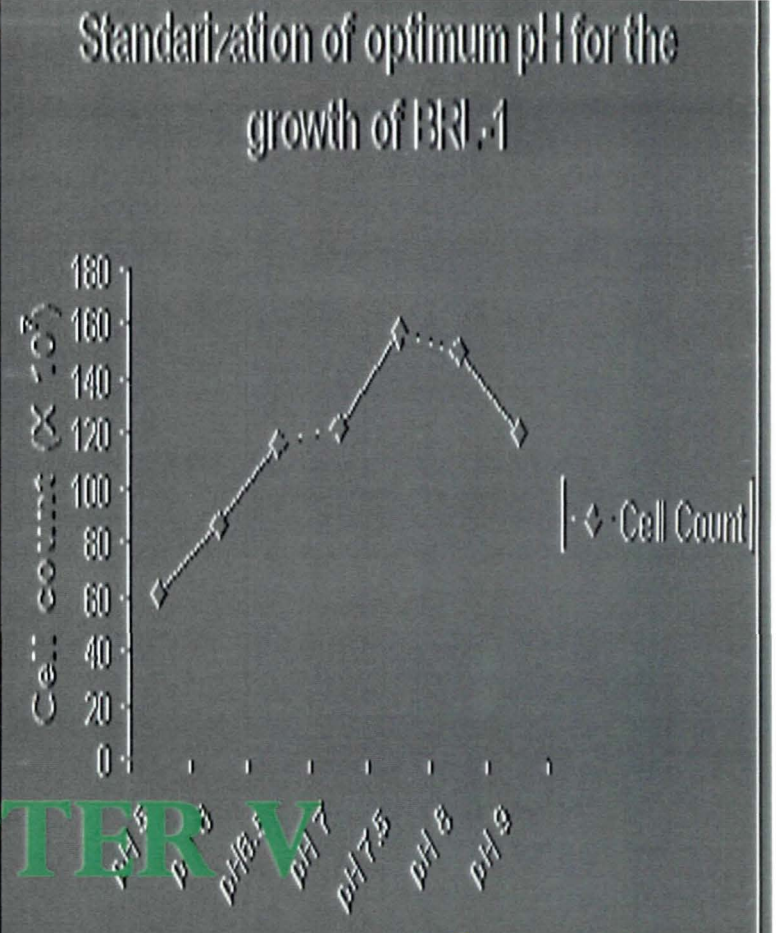
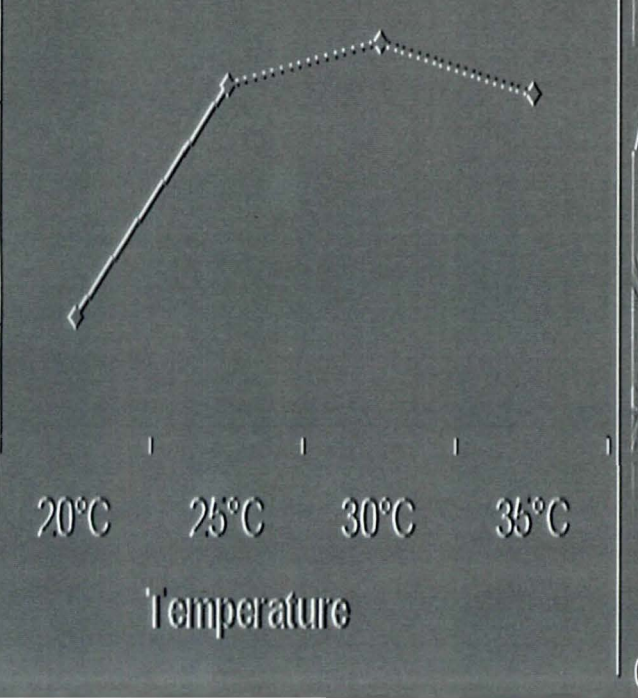
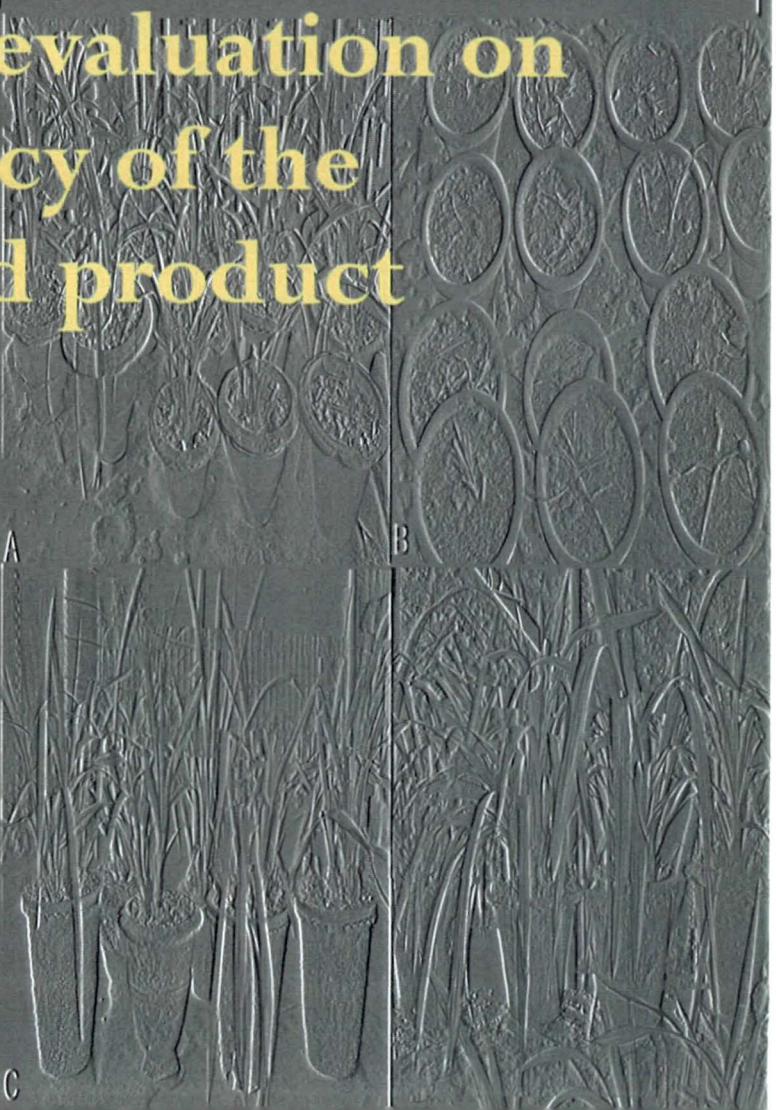




CHAPTER V

Cost effective biomass production and in vivo evaluation on the efficacy of the formulated product





5.1. Introduction

Over the past one hundred years, research has repeatedly demonstrated that phylogenetically diverse microorganisms can act as natural antagonists of various plant pathogens (Cook, 2000). The interactions between microorganisms and plant hosts can be complex. Interactions that lead to biocontrol can include antibiosis, competition, induction of host resistance and predation (Cook and Baker 1983). When testing bacterial and fungal isolates from the environment for biocontrol activities, between 1 and 10% show at least some capacity to inhibit the growth of pathogens *in vitro*. However, fewer isolates can suppress plant diseases under diverse growing conditions and fewer still have broad-spectrum activity against multiple pathogenic taxa. Nonetheless, intensive screens have yielded numerous candidate organisms for commercial development. Some of the microbial taxa that have been successfully commercialized and are currently marketed as Environmental Protection Act or EPA-registered biopesticides in the United States include bacteria belonging to the genera *Agrobacterium, Bacillus, Pseudomonas*, and *Streptomyces* and fungi belonging to the genera *Ampelomyces, Candida, Coniothyrium,* and *Trichoderma* (www.epa.gov/pesticides /biopesticides). In India bacteria like *Bacillus, Pseudomonas* and fungi like *Trichoderma* are marketed as biopesticide. Still, much remains to be learned about the microbial ecology of both plant pathogens and their microbial antagonists in different agricultural systems (Kerry, 2000; Weller *et al.*, 2002). Fundamental work remains to be done on characterizing the different mechanisms by which organic amendments reduce plant disease (Hoitink and Boehm 1999). More studies on the practical aspects of mass-production and formulation need to be undertaken to make new biocontrol products stable, effective, safer and more cost-effective (Fravel *et al.*, 1999).

Industrial interest in the development of microorganism for biological control has fluctuated over the past several decades. Once a biocontrol agent has shown potential for disease control based on laboratory, greenhouse and field tests, production of an effective biomass becomes a major concern. Advances in fermentation technology have

produced bacterial and fungal biomass for use as biocontrol insecticide and herbicides (Bowers, 1982; Churchill, 1982). However, similar technology for the production of biocontrol microorganism effective against plant pathogens is in its infancy. This deficiency is clearly an obstacle to the advancement of biocontrol research. All the biological control systems are based on a single, fundamental concept, the use of a living organism to control a pest. In order to commercialize successfully a biological control agent, this concept must be appreciated at all times. First step in the production of biocontrol agent is development of a suitable medium using inexpensive readily available agricultural by-products with the appropriate nutrient balance. (Latge and Soper, 1977). Acceptable materials include molasses, brewer's yeast, corn steep liquor, sulphite waste liquor, and cotton seed and soy flours (Lisansky, 1985).

For a successful fermentation, not only must appropriate substrates be used, but sufficient biomass containing adequate amounts of effective propagules must be obtained. Isolates of *Trichoderma* and *Gliocladium* performed best for biocontrol if preparations contained the resistant, survival propagules of the fungus i.e. chlamydospore (Lewis and Papavizas, 1984). Of various liquid media tested, small scale fermentation in molasses-brewer's yeast resulted in abundant chlamydospore production (Papavizas *et al.*, 1984). The most common equipment and processes available for the commercial production of microorganism are associated with deep tank fermentation. Deep tank fermentation has been reported by Tabachnik (1988) for the formation of conidia of *Trichoderma*. Liquid cultivation is a standard industrial method of producing cells and their products. Cultivation in batch –stirred tank reactors is the standard method of producing microbial product and such technology has been extensively developed to accommodate the food and pharmaceutical industries. Advantages of this reactor design include flexibility to accommodate multiple processes, good long-term capital investment, reliable processes and quality control, and amenability to automation and minimization of labor costs (Van Brunt, 1986). Liquid fermentation is usually employed for the production of non-obligate

pathogens such as bacteria, some fungi and nematodes (Jenkins and Goettel, 1997). There are several additional factors to consider in liquid fermentation. For example, the rate at which an effective biomass is produced affects cost of production as well as the chance of contamination and viability (Lisansky, 1985). It is desirable to obtain the optimum amount of biomass in the shortest time. With isolates of *Trichoderma, Gliocladium* and *Talaromyces,* satisfactory quantities of biomass were obtained in 6 to 7 days, but this time period is still long compared with that for bacteria. Increased fermentation time also reduces viability of some organisms and increases the risk of contamination.

Since the importance of liquid fermentation has become apparent solid state fermentation facilities have become limited in much of the world. In fact, Solid State Fermentation (SSF) has rarely been used because of insufficient consumer demand for product formed (Cannel and Moo-Young, 1980). Inspite of the rapid advances in the liquid fermentations demonstrated by industrialized nations, most of the researches on mass production of biocontrol fungi continue to emphasis solid (semi-solid) substrate fermentation for the formation of inoculum (Aidoo *et al.,* 1982). SSF are also suitable for the production of fungi which do not sporulate in liquid culture (Lisansky, 1985). New applications of SSF have been suggested for the production of antibiotics (Barrios-Gonzales *et al.,* 1988) secondary metabolites (Trejo-Hernandez *et al.,* 1992, 1993) or enriched foodstuffs (Senez *et al.,* 1980). There are several limitation of SSF such as direct determination of biomass in SSF is very difficult due to problems of separation of the microbial biomass from the substrate, subject to a greater risk of contamination and may require extensive space for processing, incubation and storage.

5.2. Formulation

Once production methods, recovery procedures and storage technology have been worked out, the next step is development of appropriate formulations for field application. A biocontrol formulation with agricultural potential should possess several

desirable characteristics such as ease of preparation and application, stability, adequate shelf life, abundant viable propagules and low cost (Churchill, 1982; Lisansky, 1985). An enormous number of amendments have been utilized in experimental and commercial formulations of different biocontrol agents. In turn, these amendments can be grouped into any number of amendment types. Generally, amendments can be grouped as either carriers (fillers, extenders) or amendments that improve the chemical, physical, or nutritional properties of the formulated biomass. Selections of amendment types along with a limited number of examples of each type are shown in Table 5.1. An extensive treatment of the topic can be found elsewhere (Bernhard *et al.*, 1998). Experimental formulations of *Bacillus* spp. that have effectively reduced plant disease have included clays (Osburn *et al.*, 1995), peat and chitin (Manjula and Podile, 2001; Sid Ahmed *et al.*, 2003), methylcellulose (Racke and Sikora, 1992), Ca-alginate, alginate manucol, or carob (Schmidt *et al.*, 2001), carboxymethyl cellulose, vegetable oil, and polyvinyl pyrrolidone (Kanjanamaneesathian *et al.*, 2000) and peptone (Ferreira *et al.*, 1991; Schmidt *et al.*, 2001) and nutrient medium (Smith *et al.*, 1993). Another various carrier-based formulation was extensively studied by Vidyasekaran and Muthamilan (1995) in case of *Pseudomonas fluorescens* shown that the bacteria can survive well in talc or peat based formulation for more than eight months.

Table 5.1. Types of amendments and example materials for formulating biocontrol biomass

Amendment type	Examples
Liquid carriers	Vegetable oils
Mineral carriers	Kaolinite clay, diatomaceous earth
Organic carriers	Grain flours
Stabilizers	Lactose, sodium benzoate
Nutrients	Molasses, peptone
Binders	Gum arabic, carboxymethylcellulose
Desiccants	Silica gel, anhydrous salts
Thickeners	Xanthan gum
Surfactants	Tween 80
Dispersants	Microcrystalline cellulose
UV protectants	
Sunscreens	Oxybenzone
Optical brighteners	Blankophor BBH
Light blockers	Lignin (PC 1307)
Stickers	Pregelatinized corn flour

To introduce an antagonist in the ecosystem and help it to survive, proliferate, become active and establish itself in a new environment, proper formulations or antagonist preparations have to be evolved. These are intended to make economically inexpensive substrate, which allow production of maximum biomass in the shortest time (Sen, 2000). Among six isolates of fluorescent *Pseudomonas* only fluorescent *Pseudomonas* BRL-1 showed effective antagonistic activity against all the three pathogens of *Cymbidium* pseudobulb rot as described earlier and also produce significant amount of indole acetic acid (IAA). Because of its potentiality the main objectives are (i) formulation of a cost effective media (ii) powder formulation using different inert carriers (iii) survibility of the organism in the inert carriers and finally (iv) *in vivo* application of inert carrier based formulated fluorescent *Pseudomonas* BRL-1.

5.3. Materials and Methods

5.3.1. Standardization of optimum condition: Conditions like method of cultivation, incubation period, pH, temperature, aeration for large scale biomass production of the selected strain BRL-1 of fluorescent *Pseudomonas* was performed utilizing a very common media (PS media) (Peptone-1%; Skim milk-1%; K_2HPO_4, 0.15% and $MgSO_4$ 0.15%) used for liquid cultivation of fluorescent pseudomonads. The biomass production was determined optically and further verified by hemocytometer cell counting method on Leica DSML research microscope. In all cases inoculum concentration was maintained at $1.2 \pm 0.25 \times 10^7$ cells / ml i.e. this would be the cell count at 0 hours in the fermentation media.

5.3.1.1. Effect of method of cultivation on biomass production: Biomass production was carried out with stationary and shake flask method (Mukherjee and Majumdar, 1971) in 100 ml Erlenmeyer flask containing 25 ml of (PS media), inoculated with 12 hours old culture of fluorescent *Pseudomonas* BRL-1. Initial inoculum concentration of the PS was set at $1.42 \pm 0.003 \times 10^7$ cells / ml, incubated at 30^0C. In the stationary method the inoculated broth were kept as such without any shake (static) where as in shake flask method the broth cultures were placed in a rotary shaker at 150 r.p.m. The biomass production was determined after different time intervals.

5.3.1.2. Effect of initial pH of the media on biomass production: Initial pH of the medium was adjusted to 5, 6, 6.5, 7, 7.5, 8 and 9 with 0.1 N HCl or NaOH. The fermentation was carried out in standardized method of cultivation in 100 ml Erlenmeyer flasks each containing 25 ml of PS inoculated with 12 hours old culture of BRL-1. The biomass production was estimated after standardized incubation time.

5.3.1.3. Effect of temperature on biomass production: To determine the optimum temperature for the large scale biomass production fermentation was carried out in standardized method of cultivation and pH at different temperatures such as 20^0C, 25^0C, 30^0C and 35^0C. Culture was taken after standardized incubation period for the assay of highest biomass yield (Mukherjee and Majumdar, 1971).

5.3.1.4. Effect of the initial number of cell in the PS Media on the biomass production: Start point cell number plays an important role for shortening of lag phase and there by increasing the biomass yield in short period. To obtain the optimum initial cell number, fermentation was carried out in 100 ml Erlenmeyer flask containing 25 ml of PS where start point cell number was varied from 0.5×10^7 to 5×10^7 cells / ml utilizing all earlier standardized parameters. Biomass yield was measured after proper incubation period.

5.3.1.5. Effect of aeration of biomass production: Effect of aeration was studied by placing different volume of medium; 15 ml 20 ml, 25 ml, 30 ml, 35 ml, 40 ml and 45 ml in 100 ml of Erlenmeyer flasks utilizing the standardized parameters. The cellular mass yield was measured after standardized incubation period.

5.3.2. Identification of a cost effective carbon and nitrogen source: After standardization of various growth factors, cost effective carbon and nitrogen sources were investigated. For identification of cost effective carbon source various sources of carbon were taken such as glucose, sucrose, fructose, molasses, skim milk at 4% and nitrogen source as peptone at 1% (w/v). For investigation of cost effective nitrogen source various organic and inorganic nitrogen sources were taken like peptone, yeast flour, mustard cake extract, fishmeal, urea, soybean flour, ammonium nitrate, ammonium chloride, ammonium sulphate, sodium nitrate and gelatin amended with selected carbon source (4% w/v).

5.3.3. Formulation of media: Finally C/N ratio was established for designing of a cost effective suitable, commercial media. As the carbon to nitrogen ratio of the media plays a very important role in biomass production investigation was next made to determine optimum C/N ratio of media using standardize carbon and nitrogen source. For this study first the cost effective standardized carbon source at fixed concentration was taken but amount of standardized nitrogen source was varied to obtain to desired C/N ratios. Further the nitrogen source at optimum ratio was fixed but amount of carbon source was varied to get the desired C/N ratio.

5.3.4. Survivility test: To test the survivility of fluorescent *Pseudomonas* BRL-1, the strain was cultured on formulated liquid broth utilizing standardized growth condition. The survival was tested in different carriers like peat, talc, farmyard manure and vermiculite. Ten grams of carboxymethyl cellulose was added to 1 kg of the carrier and mixed well. The pH was adjusted to 7.0 by adding calcium carbonate. The carriers were autoclaved twice for 30 minutes on two consecutive days. One kg of the carrier was inoculated with 400 ml of the bacterial suspension containing 8 x 10^9 cfu / ml was mixed well under sterile conditions and kept in sealed polythene bags at room temperature (22 ± 2^0C). Samples were drawn at intervals up to six months and the bacterial population was assessed by serial dilution method. Treatments were replicated three times and Petri dishes were incubated at 30^0C. After 48 hours, colony-forming units of the bacterium was recorded.

5.3.5. *In vivo* application of selected inert carrier formulation of fluorescent *Pseudomonas* BRL-1: *In vivo* evaluation of inert carrier based formulation of fluorescent *Pseudomonas* BRL-1 was performed in a polyhouse at Darjeeling. *In vivo* experiment was performed by slightly modified method of Vidhyasekaran and Muthamilan (1995). Healthy young plantlets of same age

were collected from different nurseries of several localities in Darjeeling and its adjoining areas. Collected samples were washed thoroughly with tap water and potted into earthen pots. Each pot was filled with 3 kg unsterilized soil (3 part loam and 1 part well composed farmyard manure). Experimental sets having five replicas were exposed in the polyhouse for *in vivo* experiment (Table 5.2).

Table 5.2. In vivo arrangement of pots for various treatments

Set No	Treatment
Set I	Bulbs along with roots were dipped in mixture of spore and cell suspension of all three pathogens responsible for *Cymbidium* rot.
Set II	Bulbs along with roots were dipped in mixture of spore and cell suspension of all three pathogens and uniformly coated with powdered formulation of fluorescent *Pseudomonas* BRL-1.
Set III (Control)	Bulbs were coated with powdered formulation of fluorescent *Pseudomonas* BRL-1 only.
Set IV (Blank)	Bulbs were treated with sterile distilled water only.

- *Set I*: In this set, the healthy pseudobulbs along with roots were dipped in mixture of spore / cell suspension (10^6 spores / cell /ml) of all three pathogens for two minutes and then the bulbs were potted in earthen pots.
- *Set II*: The pseudobulbs were dipped in mixture of all three pathogens (10^6 spores / cell / ml) and these dipped bulbs were then uniformly coated with powered formulation of fluorescent *Pseudomonas* BRL-1 (about 10^7 cfu/gm of powder) supplemented with 1% Carboxy Methyl Cellulose (CMC) as adhesive and planted in earthen pots.

- *Set III*: This was a control set, where bulbs were coated with powdered formulation of fluorescent *pseudomonas* BRL-1 only and transferred to earthen pot.
- *Set IV*: This was a blank set were bulbs were treated with sterile distilled water and planted in the pots.

Pots were arranged in rows, well sanitation system was maintained in the polyhouse, plants were watered regularly and disease incidence was monitored periodically.

5.4. Results and Discussion

5.4.1. Effect of method of cultivation on biomass production

Initial efforts were made to carry out the effect of method of cultivation like stationary and shake flask culture method on the growth of the organism in PS media. In both the cultivation method initial count at 0 hours was 1.42 ± 0.003 × 10^7 cells / ml. Growth was measured at different time period. Results were presented in figure 5.1.

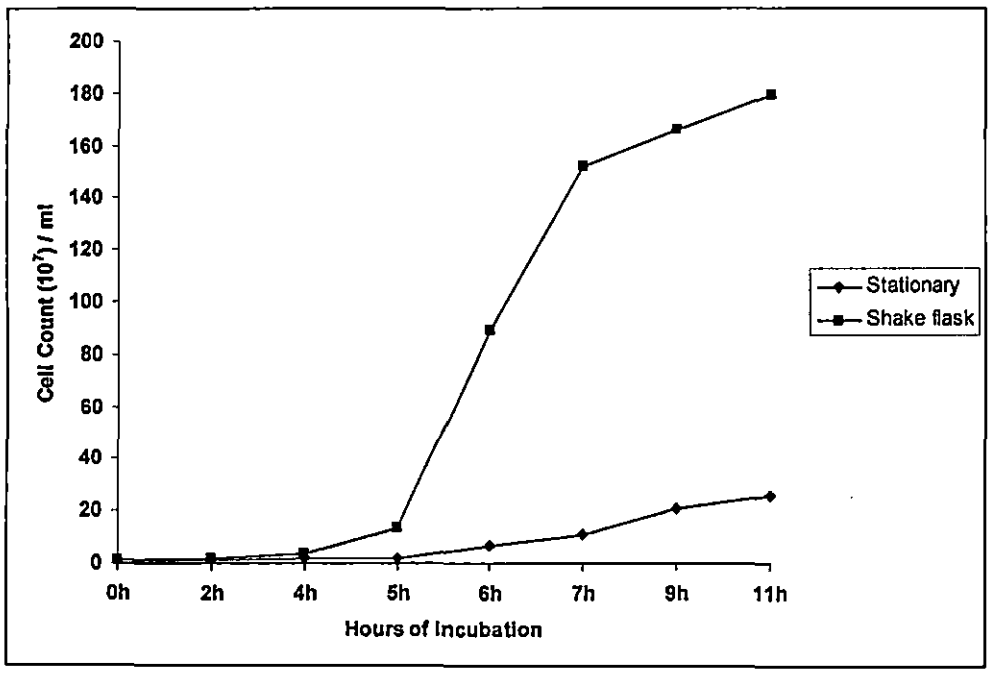


***Figure 5.1:* Effect of method of cultivation on biomass production. Results were presented mean of three separate experiments each in triplicate.**

It is evident from the figure that the shake-flaks method is superior to the stationary process. From the figure it is also evident that within 4-5th hour of incubation a sharp change in growth in shake-flask system but extended lag period was observed in stationary culture method. Furthermore, in shake-flask method cells reached to the stationary phase at short time period i.e. highest cell yield in shorter period of incubation.

5.4.2. *Effect of initial pH of the media on the biomass production*

Initial pH of the medium was adjusted to 5, 6, 6.5, 7, 7.5, 8 and 9 with 0.1 N HCl or NaOH. The fermentation was carried out in 100 ml Erlenmeyer flasks, each containing 25 ml of media. Initial count at 0 hours was 0.93×10^7 cells / ml. The broths were incubated for 7 hours at rotary shaker (150 rpm). The results were shown in Figure 5.2.

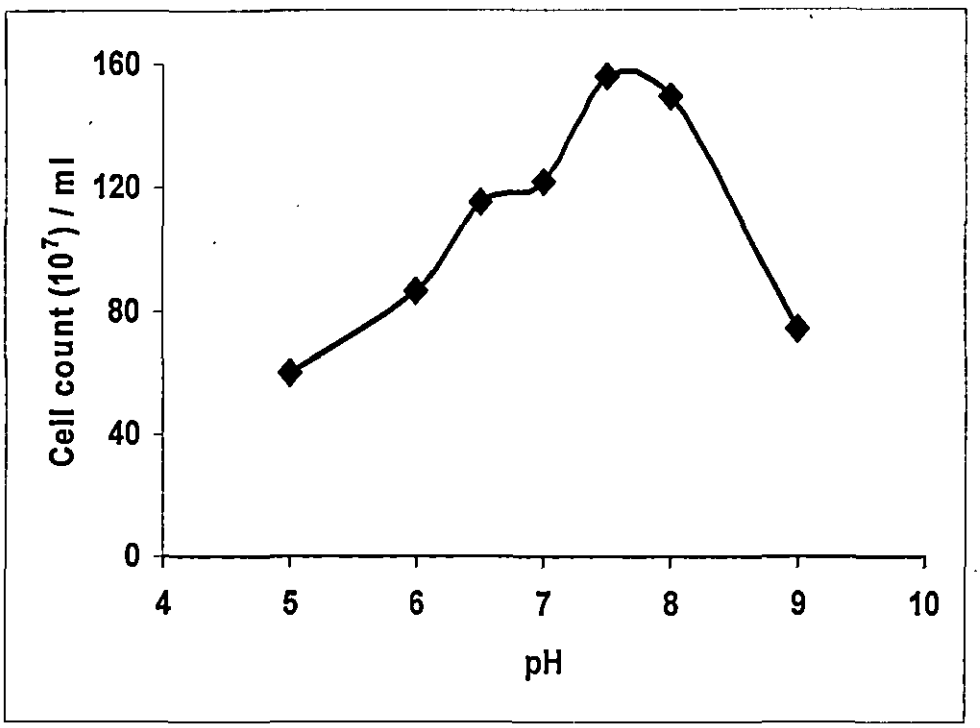


Figure 5.2: **Effect of pH on biomass production. Results were presented mean of three separate experiments each in triplicate.**

From the figure it is clear that when the initial pH of the medium was set at 7.5 the biomass yield was maximum.

5.4.3. *Effect of temperature on biomass production*

Fermentation was carried out in shake culture method at different temperatures (20^0C, 25^0C, 30^0C and 35^0C), keeping other parameters constant (incubation

period- 7 hours; method of cultivation- shake-flask; pH- 7.5) results were depicted in Figure 5.3.

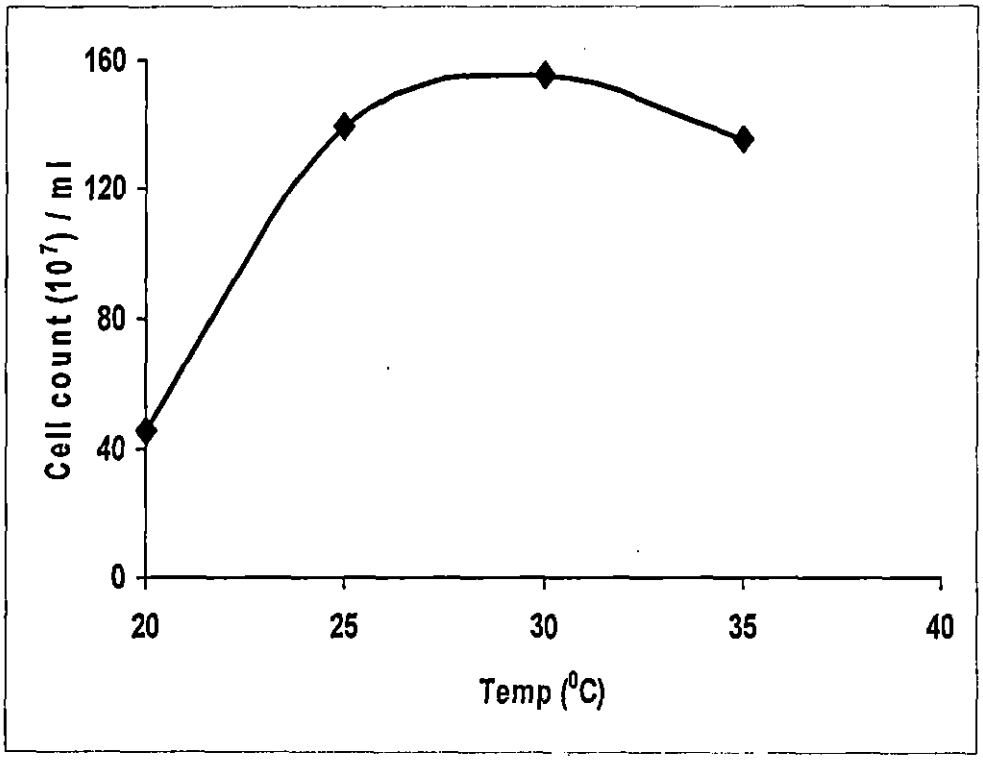


Figure 5.3: **Effect of temperature on biomass production. Results were presented mean of three separate experiments each in triplicate.**

Of the various temperatures tested for biomass yield, it was found that 30°C favoured the optimum biomass production.

5.4.4. Effect of the initial number of cell in the PS Media on the biomass production: As the start point cell number plays an important role for shortening of lag phase and there by increasing the biomass yield in short period, an attempt have been made to evaluate the role of initial cell number in the media on biomass production utilizing the earlier standardize parameters (incubation period- 7 hours; pH- 7.5; temperature- 30°C; method of cultivation- shake-flask method). Results are presented in Figure 5.4.

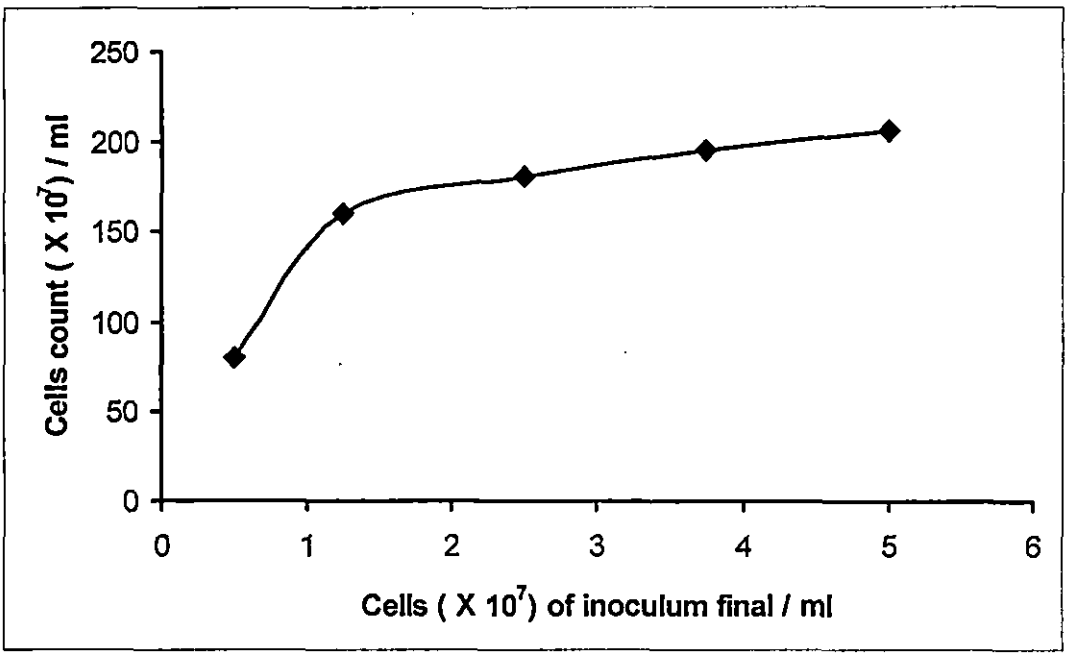


Figure 5.4: **Effect of inoculum volume of growth. Results were presented mean of three separate experiments each in triplicate.**

From the figure it is evident that the start point cell number in the PS was manipulated from 0.5 × 10^7 to 5 × 10^7 cells / ml. After the incubation period (7 hours), optimum initial cell number was found 1.25 × 10^7 cells / ml.

5.4.5. *Effect of aeration on biomass production*

Effect of aeration was studied by placing different volume of medium; 15 ml, 20 ml, 25 ml, 30 ml, 35 ml, 40 ml and 45 ml in 100 ml of Erlenmeyer flasks which were incubated for 7 hours at 30^{0}C at pH 7.5 on a rotary shaker (150 rpm). Initial number of cells in the medium was set at 1.25 × 10^7 cells /ml Degree of aeration of the fermentation broth in the shaker flask was inversely proportional to volume of liquid. The results are shown in Table 5.3.

Table 5.3. Effect of aeration on the production of biomass.

Volume of media	Cell count
15 ml	29.19×10^7
20 ml	97.6×10^7
25 ml	174.4×10^7
30 ml	817.6×10^7
35 ml	203.2×10^7
40 ml	102.4×10^7
45 ml	86.4×10^7

From the Table it is quite interesting that 30 ml of media volume in a 100 ml flask gives sudden shifting in biomass production which tempts us to speculate that adequate but optimum aeration is essential for large scale production of biomass.

After standardization of different physical factors like incubation period (7 hours), method of cultivation (shake-flaks), pH (7.5), temperature (30°C), inoculum volume (30ml) and aeration, further experiments were designed to identify a suitable cost effective carbon and nitrogen source.

5.4.6. Effect of different Carbon source on biomass production

Effect of different carbon source (4%) like glucose, sucrose, fructose, molasses, and skim milk on biomass production was determined after 7 hours of fermentation on a rotary shaker at pH 7.5 and incubated at 30°C in a total volume of 30 ml in 100 ml Erlenmeyer flask where 1% peptone used as nitrogen source in each cases. The results are shown in Figure 5.5.

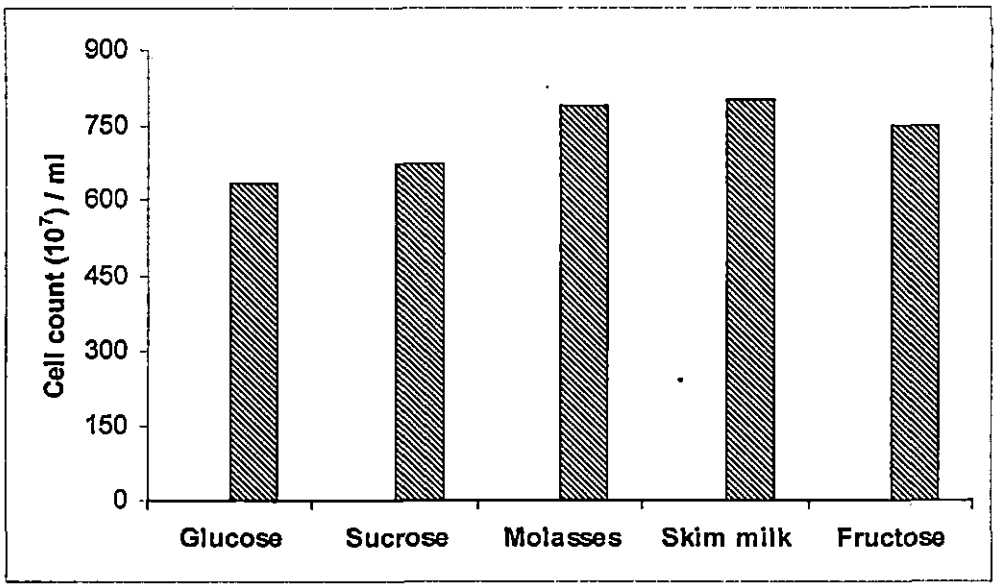


Figure 5.5: **Effect of different carbon source on biomass production. Results were presented mean of three separate experiments each in triplicate.**

Different carbon sources gave different degree of biomass yield in the fermentation broth i.e. skim milk ≅ molasses > fructose > sucrose > glucose. However, considering the cost effectiveness and the biomass production, molasses has been chosen as best carbon sources for commercial production of the strain. Now the optimum concentration of standardized carbon source i.e. molasses was determined in media ranging from 2 to 6% (Table 5.4)

Table 5.4. Optimization of concentration of Carbon source (MOLASSES) on highest biomass yield.

Carbon Source (Molasses) in %	Initial cell count /ml	Final cell Count /ml
2%	1.03×10^7	364.38×10^7
3%	1.03×10^7	556.96×10^7
4%	1.03×10^7	902×10^7
5%	1.03×10^7	668.35×10^7
6%	1.03×10^7	570.18×10^7

From this experimental result it is quite clear that at 4% concentration of molasses gave the highest yield of cellular mass.

5.4.7. Effect of different Nitrogen source on biomass production

Different nitrogen sources, including both inorganic and organic compounds were examined. To the basal medium containing 4% molasses as C source, $MgSO_4$ – 0.15%, K_2HPO_4- 0.15% and different nitrogen sources were added. Fermentation condition and the cell count method were the same as described previously. The results are shown in Figure.5.6.

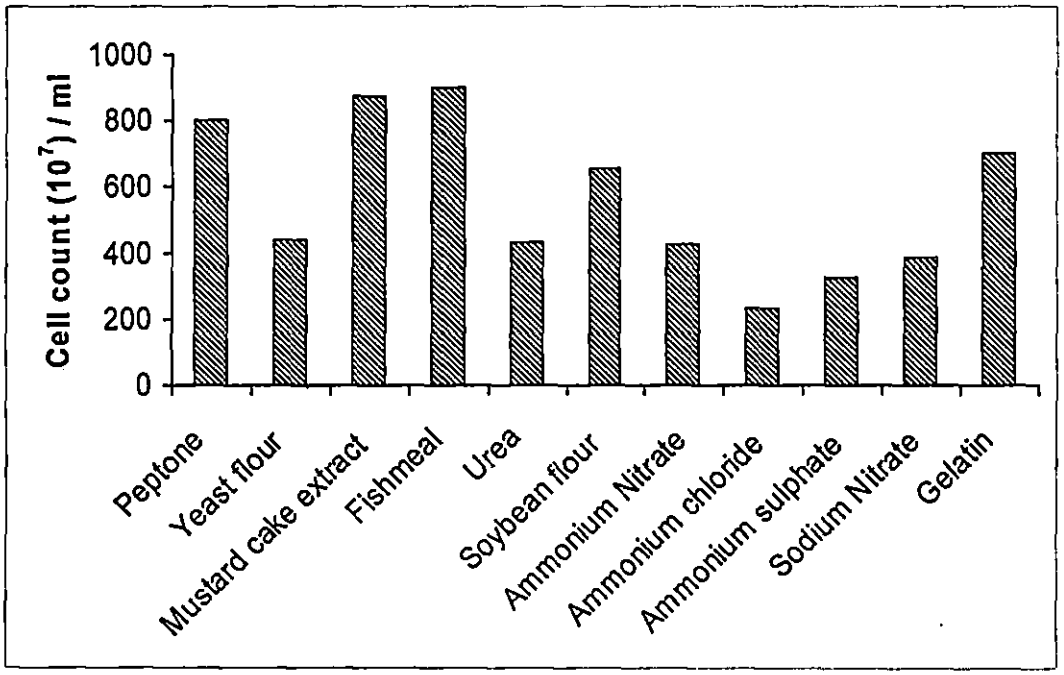


Figure 5.6: **Effect of different Nitrogen source on biomass production. Results were presented mean of three separate experiments each in triplicate.**

The figure shows that organic N_2 sources like peptone, and fishmeal were superior to inorganic N_2 sources and other organic nitrogen sources in giving higher cellular yield in said time period (7 hours). Of the various organic N_2 sources tested, fishmeal gave maximum yield and from the commercial production point of view fishmeal was the best option so it has been selected as the cheapest nitrogen source for further experiment.

5.4.8. Effect of C/N ratio on biomass production

As the carbon to nitrogen ratio of the media plays a very important role in biomass production, investigation was next made to determine optimum C/N ratio of media using molasses as carbon source and fishmeal as N_2 source. In this study molasses was used in a 4% concentration. Amount of N_2 source i.e. fishmeal was varied to obtain to desired C/N ratios. The results are indicated in Table 5.5.

Table 5.5: Optimization of C/N ratio on biomass production

C/N ratio	Cell count / ml
40	191.8×10^7
20	326.78×10^7
8	618.05×10^7
4	898×10^7
2	902×10^7

Maximum production of cells was obtained in medium with a C/N ratio of 2 but the biomass yield difference between C/N ratio 2 and 4 was very negligible and to minimize the cost of production C/N ratio at 4 has been selected as the optimum condition.

Finally, from the above experimental result a new, cost effective media was formulated with the composition of 4% molasses, 2% fishmeal, $MgSO_4$ – 0.15% and K_2HPO_4-0.15% with pH 7.5. Furthermore cost effective fermentation media formulation is not end point of biopesticide production. The next step was the development of an appropriate formulation for field application that should be inert, low cost, easy to preparation and application, should provide stability and adequate self life. Fluorescent *Pseudomonas* BRL-1 was grown in the newly formulated media, maintaining all the optimum physical condition for the strain and mixed with different types of inert, low

cost carrier like peat, talc, farmyard manure and vermiculite to check the survivility of the strain in the formulated carrier. Results were presented in the figure 5.7.

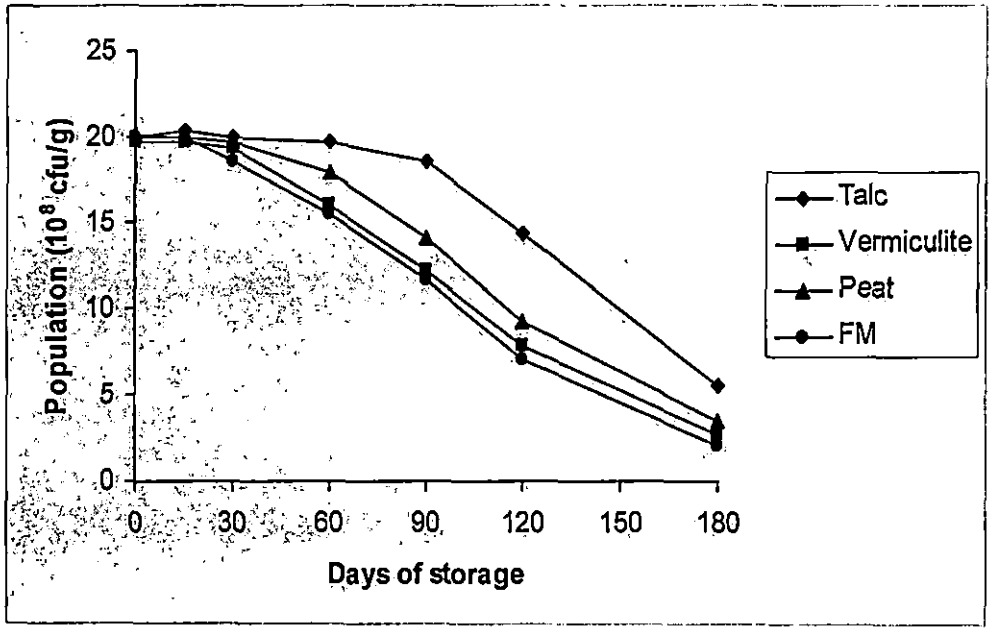


Figure 5.7: **Survivility of fluorescent *Pseudomonas* BRL-1. Results were presented mean of three separate experiments each in triplicate.**

Among the different formulated carrier for fluorescent *Pseudomonas* BRL-1, talc based formulation was the superior one. Upto 90 days the organism survived without any drastic decline from the initial population. After 6 months of storage only 4% reduction in total population was detected in talc based formulation.

5.4.9. In vivo application of inert carrier based formulation of fluorescent Pseudomonas BRL-1

The experimental sets were monitored for disease incidence upto one and half years of growth in a polyhouse at Darjeeling. All the plants of *Set I* where the bulbs were treated with the mixture of all the pathogens showed dry, fibrous, hollow pseudobulbs without any leaf and ultimately causing death of the plants (Figure 5.8 B).

Figure 5.8. In vivo experiments: (A) Blank Set: pseudobulbs were treated with sterile distilled water; (B) Bulbs were dipped in mixture of all three pathogens; (C) Treated Set: Bulbs were dipped in mixture of three pathogens and uniformly coated with talc based formulation of fluorescent Pseudomonas BRL-1; and (D) Control Set: Bulbs were coated with powdered formulation of the antagonist.

From the survivility test it was evident that talc based formulation was superior to other inert carrier so talc based formulation of fluorescent *Pseudomonas* BRL-1 was used for *in vivo* application.

In comparison to *Set I* (when bulb were dipped in the mixture of three pathogens the disease severity was maximum causing death of the plant) in *Set II* where bulbs were dipped in the mixture of three pathogens and uniformly coated with the talc based formulation of the antagonist, the plants were not only protected from the disease significantly but also showed healthy and vigorous growth (Figure 5.8 C). Uprooted plantlets of this set showed normal and healthy condition of the pseudobulbs, roots were well developed and leaves were green in colour.

In *Set III* which was the control set where the bulbs were treated with talc based formulation of fluorescent *Pseudomonas* BRL-1 only, the results showed enhanced growth both in shoot and root system (Figure 5.8 D). Plants of these set and of *Set II* showed more vigourous growth than that of the blank set (*Set IV)* (Figure 5.8 A). It has been seen that plant growth promoting rhizobacteria (PGPR) increase plant growth in various ways one of them is by releasing phytohomones (Arshad and Frankenberger, 1991; Beyeler *et al.*, 1999). The luxuriant growth of the plants of *Set II* and *Set III* could be explained by the fact that production of significant amount of phytohormone (Indole Acetic Acid) by the strain fluorescent *Pseudomonas* BRL-1.

5.5. References

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