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Microbiology

Evaluation of different Substrates for Mass Multiplication of *Pseudomonas fluorescens* in two Incubation Temperature

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Abstract

The present investigation was carried out to different organic substrate for develop simple, cheap and effective method to suitable mass multiplication of biocontrol agent Pseudomonas fluorescens. Different organic substrates were treated by sterilizated method such as hot water, steam and chemical treatment at two incubation temperature separately. 200 g of each substrate was filled in polythene bag and 48 h KMB slant culture of P. fluorescens cell suspension of concentration of 108 cfu/ml was pipetted into the each bag. The observations were recorded on 7th, 14th, 21st, 28th, and 35 th DAI. One gram of substrate from each bag was used for assessment of difference in growth of P. fluorescens and data regarding number of colony forming units (cfu) of bioagent per gram of each substrate. After 35 DAI, found that significantly higher population in FYM followed by vermi compost and gram straw at both temperature (20-25°C) and (35-40°C) irrespective of three sterilization methods taken in the study and also observed that overall growth of isolate of P. fluorescens was maximum in steam followed by hot water and least in chemical method of sterilization. As far as method of sterilization was concerned, the trend was similar as in cooler and room temperature. In all, cooler temperature (20-25°C) supported the mass multiplication over the room temperature (35-40°C).

Highlights

- Evaluate organic substrate for suitable mass multiplication of *P. fluorescens* which was sterilizated by different treatment in two incubation temperature after 35 th DAI showed maximum growth in FYM which was treated by steam at both incubation temperature.

Keywords: P. fluorescens, substrate-carriers, sterilization, incubation, biocontrol, in vitro.

The use of biological control agents as an alternative to fungicides is increasing rapidly in the present day agriculture due to the deleterious effects of chemical pesticides. Among these the bacterial antagonists have the twin advantage of faster multiplication and higher rhizosphere competence hence, P. fluorescens have been successfully used for biological control of several plant pathogens (Ramamoorthy *et al.,* 2002) and biological control using PGPR strains especially from the genus Pseudomonas is an effective substitute for chemical pesticides to suppress plant diseases (Compant *et al.,* 2005). P. fluorescens encompasses a group of common,

Gram negative, rod shaped, non pathogenic saprophytes that colonize soil, water and plant surface environments. Since they are well adapted in soil, P. fluorescens strains are being investigated extensively for use in biocontrol of pathogens in agriculture (Ganeshan and Kumar, 2006). It is known to enhance plant growth promotion and yield and reduce severity of many diseases (Hoffland *et al.,* 1996; Wei *et al.,* 1996). The cell suspensions of P. fluorescens should be immobilized in certain carriers and should be prepared as formulations for easy application, storage, commercialization and field use. The potential P. fluorescens are formulated using different organic and inorganic carriers either through solid or liquid fermentation technologies. Straw consists mainly of polymers such as cellulose, hemicellulose, and lignin, requiring the activity of specific hydrolytic enzymes for their degradation (Lynch, 1979) and has a particularly high carbon to- nitrogen ratio, between 60 and 100 (Fog, 1988). Hence, amendment of the soil with these plant residues may have the potential of altering growth conditions for the inoculated bacteria and consequently their physiological responses.

In this context, this study was undertaken to evaluate different organic substrates for mass multiplication of the potential biocontrol agent P. fluorescens and also carried out to develop simple, cheap and effective method for mass production of P. fluorescens for field application to plant disease control.

Materials and Methods

Evaluation of organic substrates for quick mass multiplication of P. fluorescens

The main purpose to carry out this study was to provide cheaper and easily available substrate for mass multiplication of P. fluorescens viz., different types of straw-wheat straw, paddy straw, soybean straw, gram straw, rice bran, vermi compost, poultry manure, FYM, cotton cake and mustard cake were evaluated.

Different sterilization methods for the organic substrates

All the substrates were sterilized by different methods to avoid the contamination and obtained good growth of Pseudomonas fluorescens.

Sterilization by hot water treatment

All the substrates were finely chopped and soaked in 100°C boiled water for 8 hrs for sterilization. Then water was completely drained out. Rice bran, vermi compost, FYM, poultry manure, cotton cake and mustard cake were moistened with water and 200 g of each substrate was filled in polythene bag (12 x 8 cm^2) and the 48 h KMB slant culture of *P. fluorescens* cell suspension of concentration of 10^8 cfu/ml was pipetted into the each bag thoroughly mixed with the help of sterilized spoon and plugged by using cotton and nylon strings. Four replications for each treatment were maintained. These bags were incubated at room temperature (35 to 40± 1°C) and cooler temperature (20 to 25± 1°C).

Observations on the number of colony farming units (CFU) per gram were recorded at 7^{th}, 14^{th}, 21^{st}, 28^{th} and 35 days after inoculation by serial dilution agar plate technique. Population dynamics was examined by mixing 1 g of formulations aseptically with 10 ml sterile distilled water for 20 min in a rotary shaker. Serial dilutions were prepared and 0.1 ml aliquot from 10^{-5} to 10^{-8} dilutions were spread on KMB plates (Chakravarty *et al.*, 2011). After incubating the plates at 28 ± 1°C for 48 h, the cfu/g formulations were counted out of *Pseudomonas fluorescens* on different substrates by using following formula (Aneja, 2003):

$$\text{No. of } P.\ fluorescens/\text{g of the sample} = \frac{\text{No. of colonies (avg. of 3 replicates)}}{\text{Amount plated} \times \text{dilution factor}}$$

Sterilization by steam treatment

All the substrates were finely chopped and soaked in water for 8 hrs then water was completely drained out. Rice bran, vermi compost, FYM, poultry manure, cotton cake and mustard cake were moistened with water and 200 g of each substrate was filled in polythene bag (12 x 8 cm^2) and sterilized in autoclave or pressure cooker. After sterilization, polythene bag were cooled and the 48 h KMB slant culture of *P. fluorescens* cell suspension of concentration of 10^8 cfu/ml was pipetted into the each bag thoroughly mixed with the help of sterilized spoon and plugged by using cotton and nylon strings. Four

replications for each treatment were maintained, these bags were incubated at room temperature (35 to 40± 1°C) and cooler temperature (20 to 25± 1°C). Observations on the number of colony farming units (CFU) per gram were recorded at 7th, 14 th, 21st, 28 th and 35 days after inoculation by dilution plate technique. Population dynamics was examined by mixing 1 g of formulations aseptically with 10 ml sterile distilled water for 20 min in a rotary shaker. Serial dilutions were prepared and 0.1 ml aliquot from 10^{-5} to 10^{-8} dilutions were spread on KMB plates (Chakravarty *et al.*, 2011). After incubating the plates at 28 ± 1°C for 48 h, the cfu/g formulations were counted out of *Pseudomonas fluorescens* on different substrates by using the formula as described earlier.

Sterilization by chemical treatment

All the substrates were finely chopped and soaked in water containing Formaldehyde (1.35 ml) + Bavistin (0.07g) lt $^{-1}$ for 12 hrs for sterilization then water was completely drained out. Rice bran, vermi compost, FYM, poultry manure, cotton cake and mustard cake were moistened with water contains Formaldehyde (1.35 ml lt^{-1}) + Bavistin (0.07 g lt $^{-1}$). Each substrate of 200 g was filled in polythene bag (12 x 8 cm^2) and the 48 h KMB slant culture of *P. fluorescens* cell suspension of concentration of 10^8 cfu/ml was pipetted into the each bag thoroughly mixed with the help of sterilized spoon and plugged by using cotton and nylon strings. Four replications for each treatment were maintained. These bags were incubated at room temperature (35 to 40± 1°C) and cooler temperature (20 to 25± 1°C).

Observations on the number of colony forming units (CFU) per gram were recorded at 7th, 14 th, 21st, 28 th and 35 day after inoculation by dilution plate technique. Population dynamics was examined by mixing 1 g of formulations aseptically with 10 ml sterile distilled water for 20 min in a rotary shaker. Serial dilutions were prepared and 0.1 ml aliquot from 10^{-5} to 10^{-8} dilutions were spread on KMB plates (Chakravarty *et al.*, 2011). After incubating the plates at 28 ± 1°C for 48 h, the cfu/g formulations were counted out of *Pseudomonas fluorescens* on different substrates by using the formula as described earlier.

Results and Discussion

The present investigation was carried out to screen different organic substrate suitable for mass multiplication and sterilizated by hot water, steam and chemical treatment. These were incubated at two incubation temperatures. One gram of substrate from each bag was used for assessment of difference in growth of *Pseudomonas fluorescens*. The observations were recorded on 7, 14, 21, 28, and 35 DAI and data regarding number of colony forming units (cfu) of bioagent per gram of each substrate are presented in Table 1 and 2 and graph 1-6.

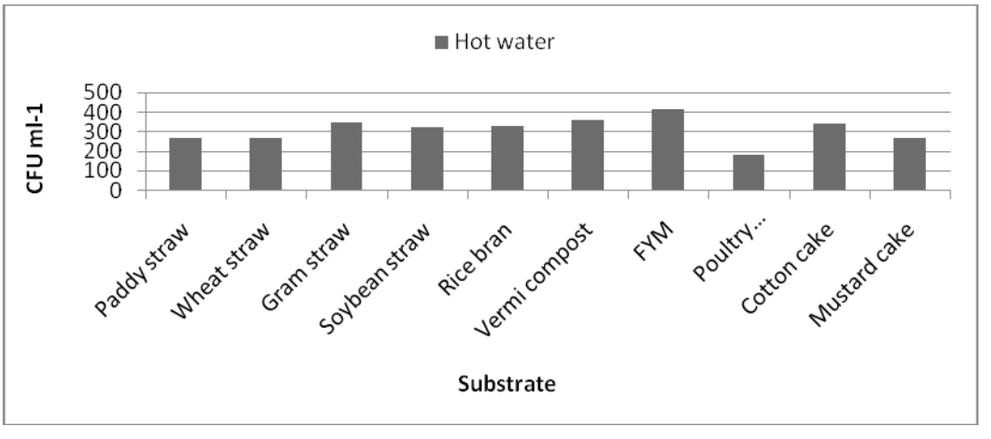

Fig. 1. Effect of hot water sterilization method on mass multiplication of Pseudomonas fluorecsens (x 108 cfu/g) in different substrates at 20-25° C

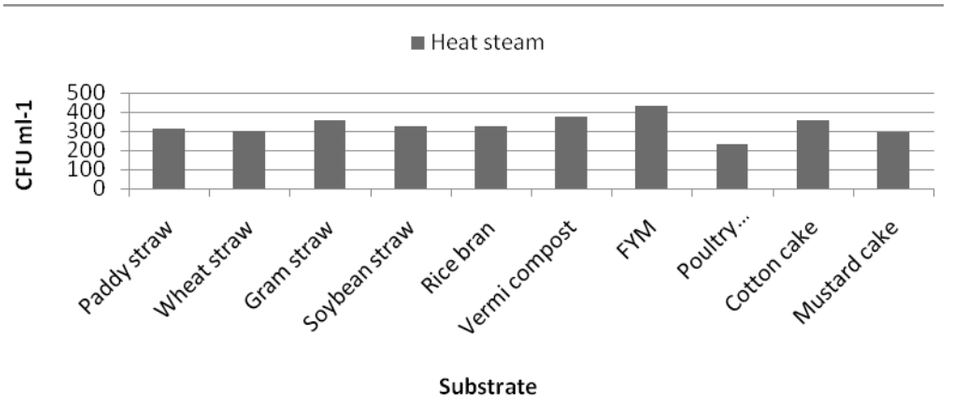

Fig. 2. Effect of heat steam sterilization method on mass multiplication of Pseudomonas fluorecsens (x 108 cfu/g) in different substrates at 20-25° C

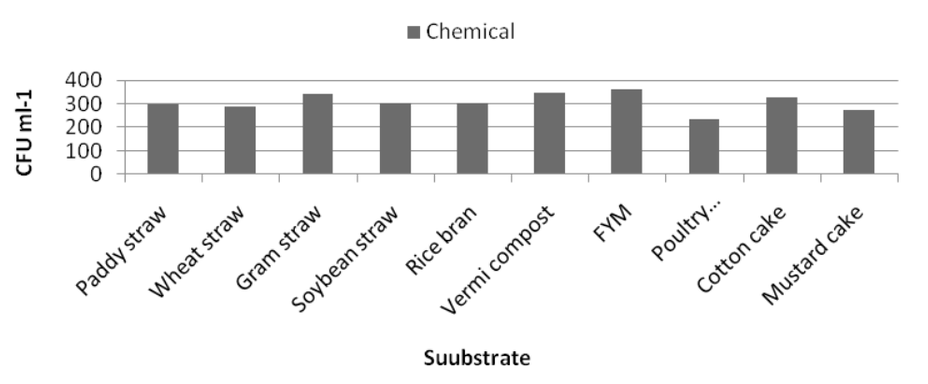

Fig. 3. Effect of chemical sterilization method on mass multiplication of Pseudomonas fluorecsens (x 108 cfu/g) in different substrates at 20-25° C

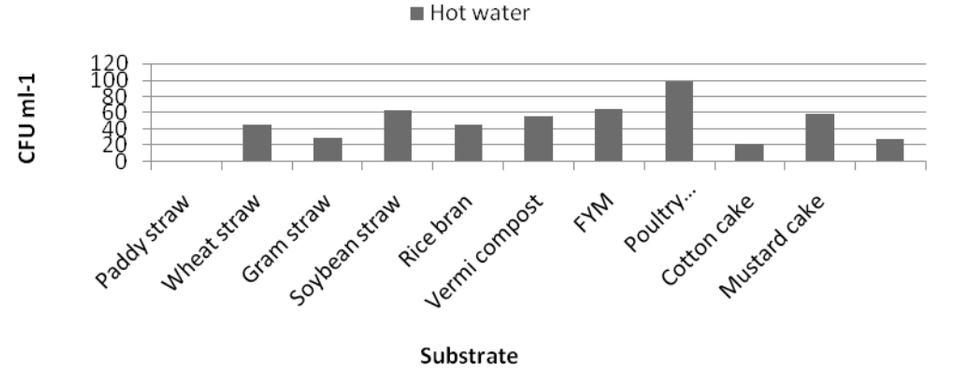

Fig. 4. Effect of hot water sterilization method on mass multiplication of Pseudomonas fluorecsens (x 108 cfu/g) in different substrates at 35-40° C

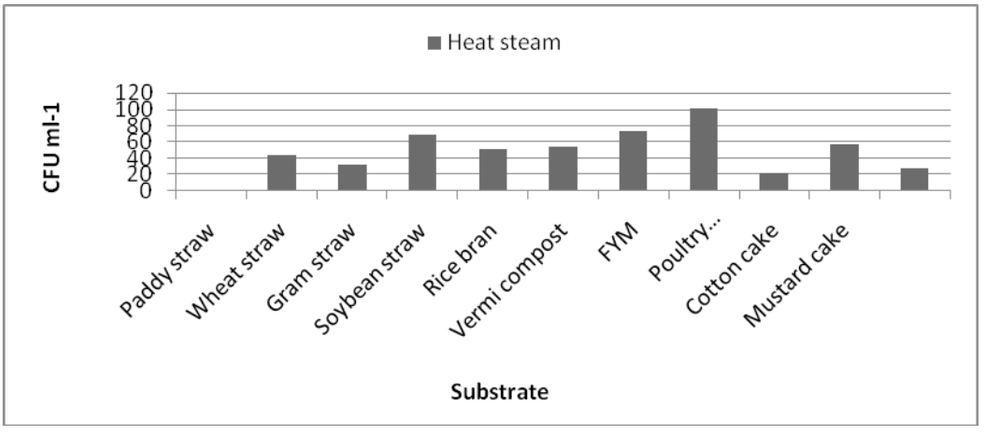

Fig. 5. Effect of heat steam sterilization method on mass multiplication of Pseudomonas fluorecsens (x 108 cfu/g) in different substrates at 35-40°C

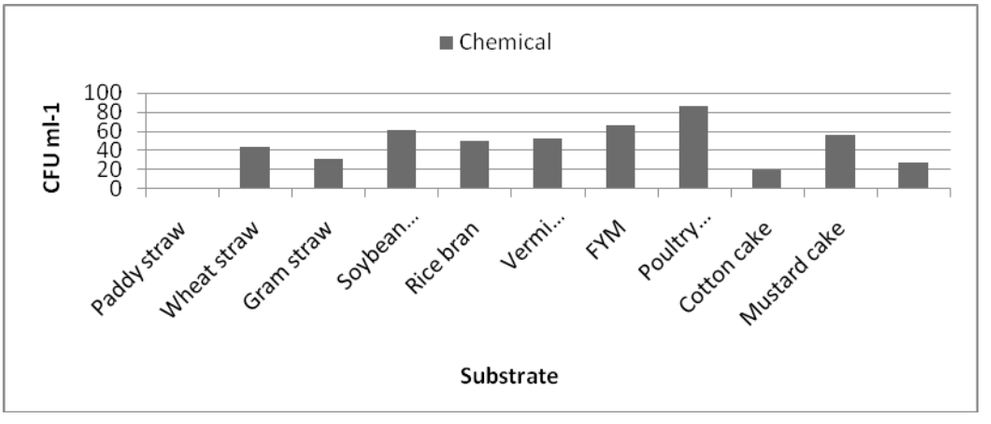

Fig. 6. Effect of chemical sterilization method on mass multiplication of Pseudomonas fluorecsens (x 108 cfu/g) in different substrates at 35-40° C

At cooler temperature (20-25°C)

Ten substrates were evaluated for three sterilization methods at cooler temperature (20-25°C) at different incubation period (7 to 35 days) for their effect on mass multiplication of local isolate of Pseudomonas fluorescens.

It is evident from the data presented in Table 1 that P. fluorescens multiplied best in substrate farm yard manure which was closely followed by vermi compost and gram straw after 7, 14, 21, 28 and 35 days of inoculation when hot water was used for sterilization. Among these top three substrates, farm yard manure, vermi compost and gram straw was significantly superior over other substrate at 7 DAI; FYM was at par with vermi compost but significantly higher to gram straw at 14 DAI; non significant difference among top three substrates at 21, 28 and 35 DAI. In rest of the substrate, population of P. fluorescens was significantly lower than three top substrates from 7 to 35 DAI.

Table 1. Effects of sterilization methods and different incubation period on mass multiplication in different substrates of *Pseudomonas fluorecsens* (x 10^8 cfu/g) at 20-25 °C

Incubated at 20-25° C																	
S. N.	Substrate	7 Days*			14 Days*			21 Days*			28 Days*			35 Days*			
		Hot water treated	Steam treated	Chemical treated	Hot water treated	Steam treated	Chemical treated	Hot water treated	Steam treated	Chemical treated	Hot water treated	Steam treated	Chemical treated	Hot water treated	Steam treated	Chemical treated	
1	Paddy straw	33.92 (9.53)**	24.42 (9.34)	37.08 (9.57)	67.00 (9.82)	61.42 (9.74)	64.83 (9.80)	101.50 (10.00)	122.92 (10.09)	105.08 (10.02)	189.67 (10.28)	215.33 (10.33)	194.58 (10.29)	269.00 (10.43)	313.50 (10.49)	298.83 (10.47)	
2	Wheat straw	27.58 (9.44)	21.67 (9.33)	31.58 (9.50)	66.67 (9.81)	59.33 (9.77)	58.08 (9.76)	81.08 (9.90)	82.83 (9.92)	92.92 (9.97)	167.25 (10.22)	197.42 (10.29)	186.67 (10.25)	266.17 (10.42)	299.58 (10.47)	287.08 (10.46)	
3	Gram straw	49.58 (9.69)	51.50 (9.71)	46.08 (9.64)	77.50 (9.89)	104.17 (10.01)	80.50 (9.90)	139.92 (10.14)	152.25 (10.18)	146.25 (10.16)	223.08 (10.35)	244.58 (10.39)	236.75 (10.37)	346.67 (10.54)	356.08 (10.55)	340.33 (10.53)	
4	Soybean straw	41.92 (9.61)	29.58 (9.47)	37.58 (9.57)	68.08 (9.82)	64.58 (9.81)	69.33 (9.84)	119.22 (10.08)	124.83 (10.10)	115.58 (10.06)	198.00 (10.30)	215.75 (10.33)	209.25 (10.32)	324.25 (10.51)	325.50 (10.51)	300.08 (10.47)	
5	Rice bran	43.50 (9.63)	35.50 (9.54)	43.08 (9.60)	73.28 (9.86)	78.00 (9.89)	73.67 (9.87)	129.92 (10.11)	126.83 (10.10)	121.50 (10.08)	214.83 (10.33)	223.33 (10.35)	212.92 (10.33)	328.25 (10.51)	325.92 (10.51)	301.08 (10.47)	
6	Vermi compost	50.33 (9.70)	60.50 (9.78)	48.58 (9.69)	100.92 (10.00)	104.42 (10.01)	85.58 (9.92)	144.67 (10.16)	162.67 (10.21)	147.67 (10.17)	246.67 (10.39)	253.00 (10.40)	237.08 (10.37)	359.42 (10.56)	376.67 (10.58)	348.75 (10.54)	
7	FYM	55.33 (9.74)	84.17 (9.92)	51.67 (9.71)	113.25 (10.05)	105.17 (10.02)	94.17 (9.97)	162.92 (10.21)	205.42 (10.31)	165.33 (10.22)	249.67 (10.40)	256.75 (10.41)	250.92 (10.40)	414.42 (10.62)	428.50 (10.63)	361.92 (10.56)	
8	Poultry manure	15.08 (9.15)	12.06 (9.02)	16.28 (9.17)	26.25 (9.37)	47.25 9.67)	29.33 (9.46)	65.25 (9.81)	70.42 (9.85)	60.25 (9.78)	119.56 (10.05)	100.83 (10.00)	114.42 (10.06)	184.33 (10.26)	230.85 (10.36)	236.24 (10.37)	
9	Cotton cake	44.75 (9.65)	43.25 (9.63)	45.75 (9.66)	75.83 (9.88)	99.67 (10.00)	78.08 (9.89)	133.92 (10.12)	141.92 (10.15)	136.08 (10.13)	217.67 (10.34)	230.50 (10.35)	233.00 (10.37)	340.92 (10.53)	354.08 (10.55)	326.17 (10.51)	
10	Mustard cake	17.75 (9.21)	9.92 (8.95)	14.25 (9.13)	55.58 (9.74)	59.17 (9.77)	50.67 (9.70)	80.50 (9.90)	78.33 (9.89)	80.83 (9.91)	157.17 (10.18)	168.75 (10.22)	149.67 (10.17)	269.00 (10.53)	291.67 (10.46)	274.83 (10.44)	
$SE_{(m)}\pm$		0.06	0.07	0.06	0.05	0.05	0.04	0.03	0.02	0.02	0.04	0.03	0.03	0.08	0.02	0.03	
CD (5%)		0.16	0.21	0.18	0.15	0.14	0.10	0.09	0.07	0.05	0.12	0.09	0.09	0.22	0.07	0.10	

* Average of four replications

**Figures within parentheses indicate log transformed values

Cfu – colony forming units, DAI – Days after inoculation

Table 2. Effects of sterilization methods and different incubation period on mass multiplication in different substrates of *Pseudomonas fluorecsens* (x 10^8 cfu/g) at 35-40°C

Incubated at 35-40 °C																
S. N.	Sub-strate	7 Days*			14 Days*			21 Days*			28 Days*			35 Days*		
		Hot water treated	Steam treated	Chemical treated	Hot water treated	Steam treated	Chemical treated	Hot water treated	Steam treated	Chemical treated	Hot water treated	Steam treated	Chemical treated	Hot water treated	Steam treated	Chemical treated
1	Paddy straw	39.42 (9.60)**	44.67 (9.65)	36.83 (9.54)	59.58 (9.77)	66.58 (9.82)	64.58 (9.80)	103.25 (10.01)	111.83 (10.05)	104.17 (10.02)	64.33 (9.81)	57.50 (9.76)	59.50 (9.77)	44.83 (9.65)	43.83 (9.64)	44.34 (9.64)
2	Wheat straw	34.42 (9.53)	43.50 (9.64)	35.92 (9.55)	57.83 (9.76)	58.50 (9.76)	63.58 (9.80)	96.50 (9.97)	107.83 (10.03)	88.67 (9.93)	48.75 (9.68)	56.17 (9.75)	54.50 (9.73)	28.83 (9.46)	32.92 (9.51)	31.17 (9.49)
3	Gram straw	44.50 (9.64)	53.42 (9.73)	45.08 (9.65)	72.08 (9.86)	79.50 (9.90)	71.25 (9.85)	111.25 (10.04)	144.08 (10.15)	117.33 (10.07)	159.50 (10.20)	156.08 (10.19)	143.92 (10.16)	63.58 (9.80)	69.42 (9.84)	61.83 (9.79)
4	Soybean straw	42.17 (9.62)	48.17 (9.68)	38.75 (9.59)	65.83 (9.81)	70.14 (9.85)	64.43 (9.81)	103.08 (10.01)	114.17 (10.06)	110.83 (10.04)	69.92 (9.84)	79.58 (9.89)	64.58 (9.80)	45.83 (9.66)	50.75 (9.70)	49.50 (9.69)
5	Rice bran	43.75 (9.64)	48.67 (9.68)	41.58 (9.61)	67.42 (9.83)	70.58 (9.85)	69.67 (9.84)	106.50 (10.00)	114.92 (10.06)	113.83 (10.05)	76.75 (9.88)	94.42 (9.97)	93.25 (9.97)	55.33 (9.74)	53.50 (9.73)	52.42 (9.72)
6	Vermi compost	51.42 (9.71)	57.17 (9.75)	49.75 (9.69)	75.50 (9.88)	85.08 (9.93)	77.92 (9.89)	112.58 (10.05)	215.33 (10.33)	119.42 (10.08)	177.83 (10.25)	188.83 (10.27)	177.08 (10.24)	63.92 (9.80)	72.92 (9.86)	66.75 (9.82)
7	FYM	51.92 (9.71)	61.67 (9.79)	52.17 (9.72)	80.17 (9.90)	106.67 (10.03)	79.50 (9.90)	112.83 (10.05)	228.50 (10.35)	123.25 (10.09)	180.25 (10.25)	239.59 (10.38)	180.25 (10.25)	98.00 (9.99)	100.67 (10.00)	86.84 (9.93)
8	Poultry manure	18.09 (9.23)	34.92 (9.53)	15.58 (9.15)	56.33 (9.75)	56.92 (9.75)	49.42 (9.69)	84.00 (9.92)	82.25 (9.90)	71.00 (9.84)	43.58 (9.63)	44.50 (9.65)	42.42 (9.63)	21.67 (9.33)	21.75 (9.33)	19.50 (9.29)
9	Cotton cake	43.83 (9.64)	49.42 (9.69)	44.33 (9.64)	68.25 (9.83)	71.75 (9.85)	69.83 (9.84)	107.17 (10.03)	116.00 (10.06)	116.33 (10.06)	118.08 (10.07)	133.42 (10.12)	109.00 (10.04)	58.83 (9.77)	56.50 (9.75)	56.83 (9.75)
10	Mustard cake	29.25 (9.47)	35.42 (9.55)	27.75 (9.43)	57.33 (9.76)	57.33 (9.76)	54.33 (9.73)	96.25 (9.98)	107.75 (10.03)	85.67 (9.93)	48.25 (9.68)	49.08 (9.69)	44.00 (9.64)	27.25 (9.43)	27.75 (9.44)	27.17 (9.43)
$SE_{(m)}\pm$		0.04	0.03	0.06	0.03	0.02	0.03	0.04	0.03	0.04	0.03	0.03	0.03	0.03	0.03	0.04
CD (5%)		0.11	0.10	0.16	0.08	0.07	0.08	0.12	0.08	0.11	0.08	0.07	0.08	0.08	0.10	0.10

* Average of four replications

**Figures within parentheses indicate log transformed values

The trend was similar for P. fluorescens population when substrates sterilized with steam and chemicals from 7 to 35 DAI. Among these top three substrates, farm yard manure, vermi compost and gram straw was significantly superior over other substrate at 7 and 14 DAI. FYM was significantly superior over other substrate at 21 DAI; non significant difference among top three substrates at 28 DAI and 35 DAI when steam was used for sterilization.

Significant higher population of P. fluorescens was observed in farm yard manure, vermi compost and gram straw at 7, 14 DAI. FYM was at par with vermi compost but significantly higher to gram straw at 21 DAI when chemicals were used for sterilization. Whereas non significant differences were observed among all these top three substrate at 28 and 35 DAI.

After 35 DAI, it was observed that overall growth of local isolate of P. fluorescens was maximum in steam followed by hot water and least in chemical method of sterilization.

Plate 1. Mass multiplication of Pseudomonas fluorescens on hot water sterilized organic substrates

At room temperature (35-40°C)

Three methods of sterilization and ten substrates were evaluated for their effect on mass multiplication of local isolate of P. fluorescens at room temperature (35- 40°C) at 7, 14, 21, 28 and 35 DAI.

The data presented in table 2 revealed that farm yard manure was found best followed by vermi compost and gram straw for mass multiplication of P. fluorescens at 7 DAI, when sterilized by hot water. This trend was continued at 14, 21, 28 and 35 DAI. At 7, 14, 21 and 28 DAI, non significant difference was observed among these three substrates. At 35 DAI, significantly higher population in FYM was recorded than that of in vermi compost and gram straw.

Steam was used for sterilization of substrates and it is clear from the presented in table 2 that farm yard manure was found best among the substrates tested followed by vermi compost and gram straw at 7 DAI. Similar trend

Plate 2. Mass multiplication of Pseudomonas fluorescens on heat steam sterilized organic substrates

was recorded at 14, 21, 28 and 32 DAI. No significant difference was observed among the top three substrates at 7 DAI. At 14, 28 and 35 DAI, significantly higher population in FYM was recorded than that of in vermi compost and gram straw. Whereas FYM was at par with vermi compost and significantly superior over gram straw at 21 DAI. FYM was found best among the substrates tested followed by vermi compost and gram straw at 7 DAI when chemicals were used for sterilization. The trend was continued at 14, 21, 28 and 35 DAI. Top three substrates were non significant among themselves at 7, 14, and 21 DAI. At 35 DAI, significantly higher population in FYM was recorded than that in vermi compost and gram straw. Whereas FYM was at par with vermi compost and significantly superior over gram straw at 28 DAI.

The population dynamics of P. fluorescens at 35- 40°C showed that there was slow and progressive decline on 28 and 35 DAI in all sterilization methods.

As far as method of sterilization was concerned, the trend was similar as in cooler temperature. In all, cooler temperature (20-25°C) supported the mass multiplication over the room temperature (35-40°C).

Several workers reported various substrates suitable for mass multiplication of P. fluorescens. Bora and Deka (2007) found vermicompost; Jayaraj *et al.,* (2007) found poultry manure and FYM; Ambardar and Sood (2010) reported FYM + sand and Chakravarty and Kalita (2011) recorded vermicompost and FYM as host substrates for P. fluorescens multiplication. Almost similar results were also obtained in present study agreeing with the findings of earlier workers. On the contrary, Niranjana *et al.,* (2009) reported rice bran was host substrate but in the present findings, rice bran substrate was not reported the growth of P. fluorescens on compared to others.

Conclusion

Hence, it is concluded that out of ten substrates tested for suitability to mass multiplication of local isolate of Pseudomonas fluorescens and it was found that FYM supported maximum growth followed by vermi compost and gram straw at cooler temperature (20-25°C) irrespective of three sterilization methods taken in the study. After 35 DAI, it was observed that overall growth of isolate of P. fluorescens was maximum in steam followed by hot water and least in chemical method of sterilization. The trends for substrate and method optimization were similar at room temperature (35-40°C). Therefore In all, cooler temperature (20-25°C) supported the more mass multiplication over the room temperature (35-40°C).

Plate 3. Different organic substrate sterilized by chemical for mass multiplication of P. fluorescens

Plate 4. Observed plates of different substrate (a) Paddy straw, (b) Wheat straw, (c) Gram straw, (d) Soybean straw, (e) Rice bran, (f) Vermi compost, (g) FYM, (h) Poultry manure, (i) Cotton cake, (j) Mustard cake.

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