CHAPTER IV

RESULTS

4.1 Isolation of bacteria

The bacteria used in this research were taken from various sources. First, 27 strains of *Bacillus subtilis* were provided by N. Maneechote (2001) and 16 isolates of denitrifying bacteria were given from K. Smartivutikoon (2003). Other sources of bacteria included, the soil around food waste dumping area, decomposed vegetables, soil around hot spring areas from Sankampang, Chiang Mai, Thailand, and finally, bacteria obtained from the compost of The Royal Project, Chitralada Palace.

All samples were screened for bacteria capable of producing three enzymatic activities namely, cellulase, protease and lipase.

4.1.1 Isolation of cellulase producing bacteria

The cellulose degrading bacteria were screened using the CMC agar plates and the results were depicted in Figure 4.1. The ratios of the clear zones around the colonies were tabulated in Figure 4.2. From this results, the colonies of the bacteria with the maximum clear zones ratio were isolated from The Royal Project compost including: P1, P2, P3, Y1 and Y2. These bacterial isolates were selected for further enzyme activity measurement.

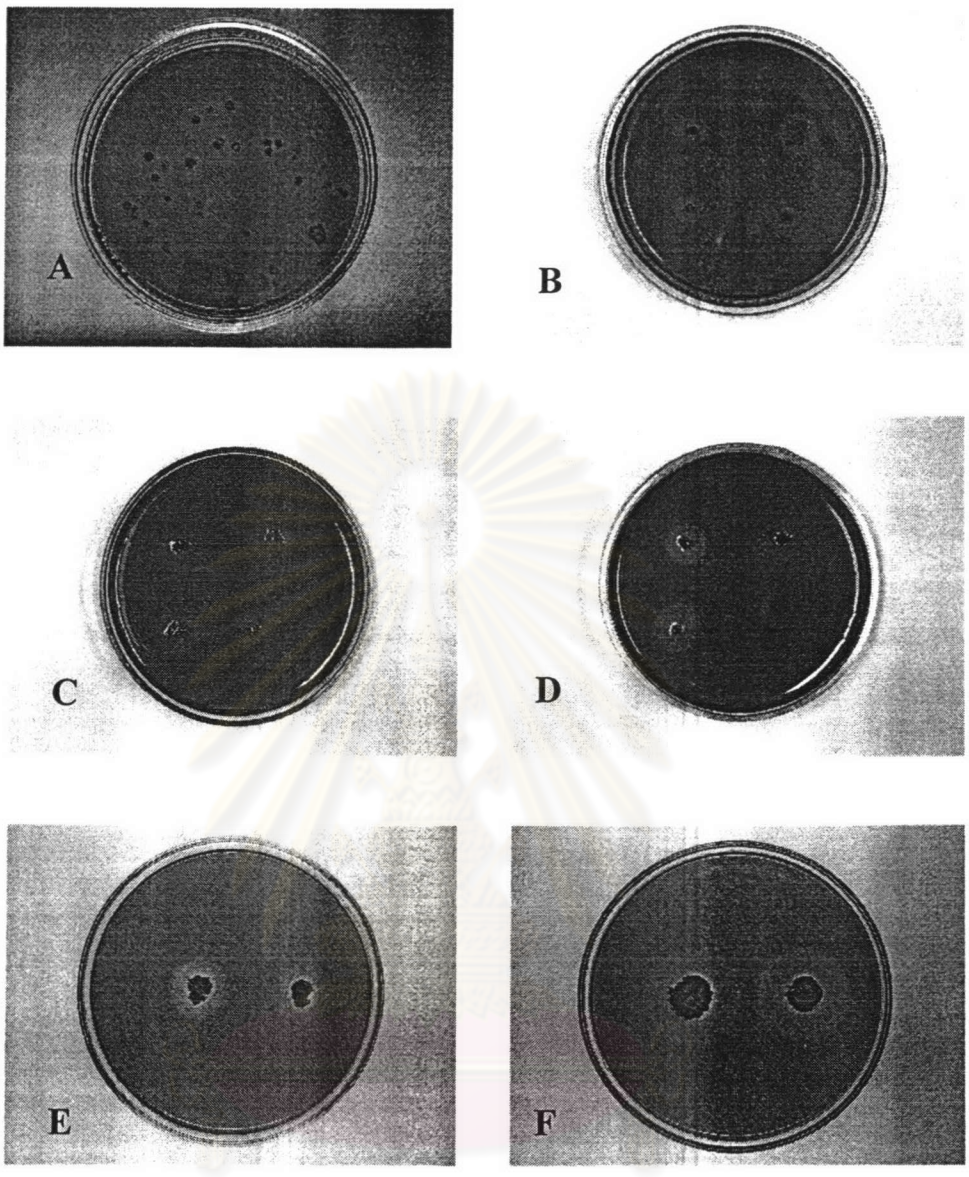

Figure 4.1 The clear zones produced around the selected bacterial colonies growing on CMC agar stained with congo red: A) serial dilution of sample spread on CMC agar plate; B) 4 strains of *B. subtilis*; C-D) bacterial isolates from decomposed vegetable; E- F) bacterial isolates from The Royal Project compost.

From Figure 4.1, when 27 isolates of *Bacillus subtilis* (N1-N27), 7 isolates of bacteria from decomposed vegetables (A1-A4, B1-B3) and 5 isolates of bacteria from The Royal Project compost were screened for cellulase production, 5 isolates of highest clear zone producing strains, P1, P2, P3, Y1, Y2, were selected for cellulase activities.

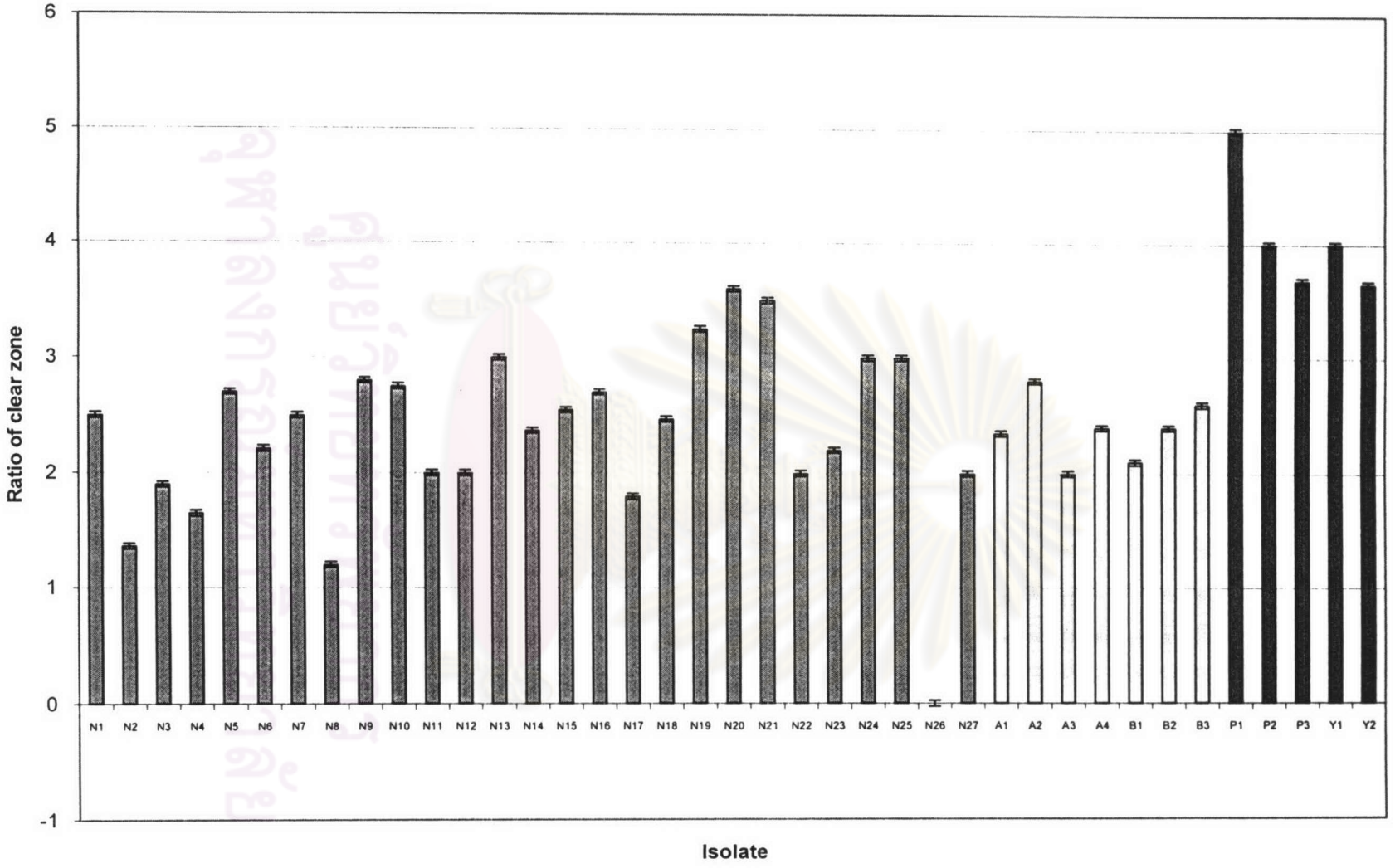

Figure 4.2 The ratio of clear zones of bacteria isolated from various sources after growing on CMC agar plates for 1 day and stained with congo red: bacterial from N. Maneechote (N1-N27), decomposed vegetables (A1-A4, B1-B3) and The Royal Project compost (P1-P3, Y1-Y2).

4.1.2 Isolation of protease producing bacteria

Figure 4.3 showed the clear zones around the colonies of the bacteria growing on skim milk agar plates. The results revealed that, 4 isolates of *Bacillus subtilis*, namely N3, N4, N8 and N19, were found to produce the clear zones. When the samples taken from decomposed vegetables and the soil around the food waste dumping area were screened, 10 isolates, called A1, A2, A3, A4, B1, B2, B3, G1, G2 and G3, were found with the clear zones. Moreover, the protease producing bacteria were also screened from the hot spring area. The results revealed that there were 7 isolates (D1- D7) that gave the clear zones around the colonies (Figure 4.4).

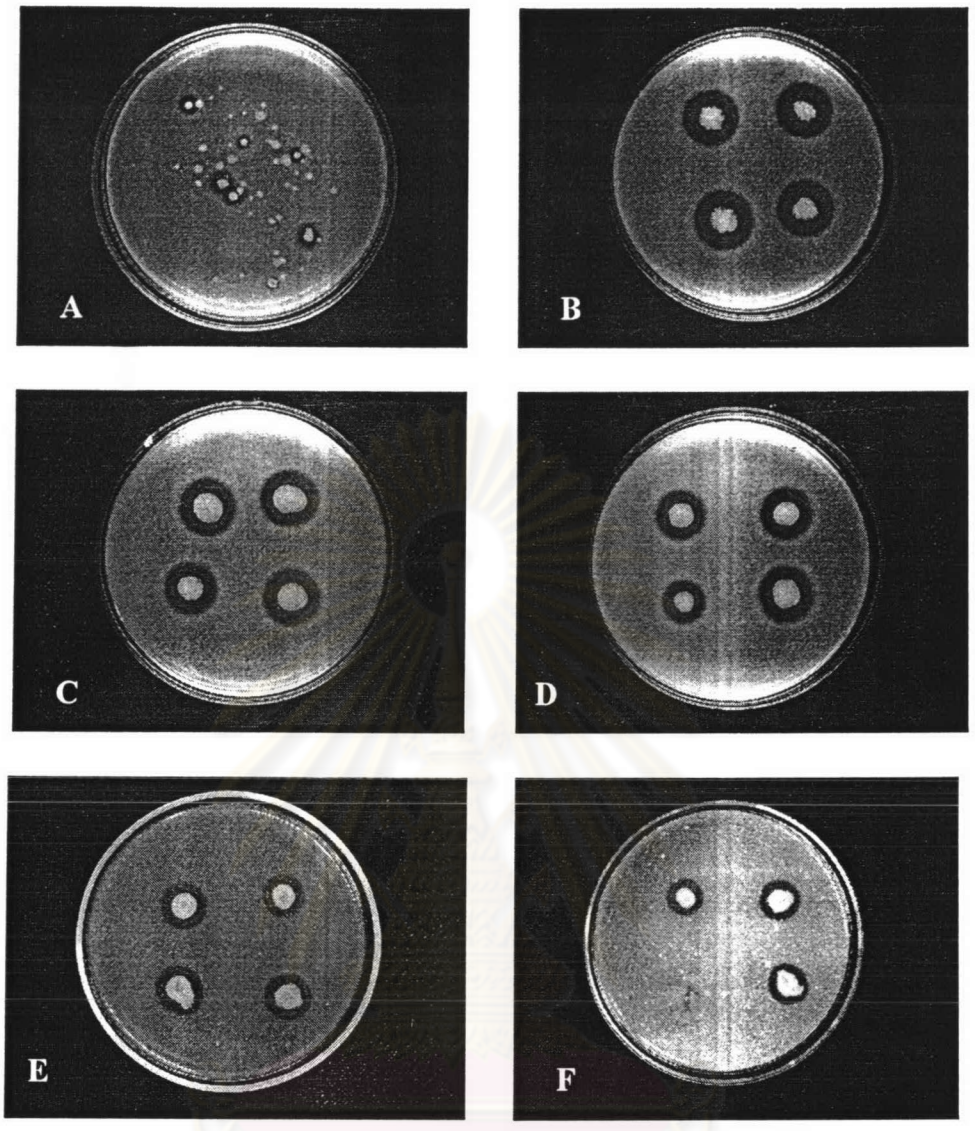

Figure 4.3 The clear zones around the selected bacterial colonies on skim milk agar plates: A) serial dilution of sample from decomposed vegetable; B) *B. subtilis* isolates; C-D) bacteria from decomposed vegetables; E) bacteria from hot spring area; F) bacteria from food waste dumping area.

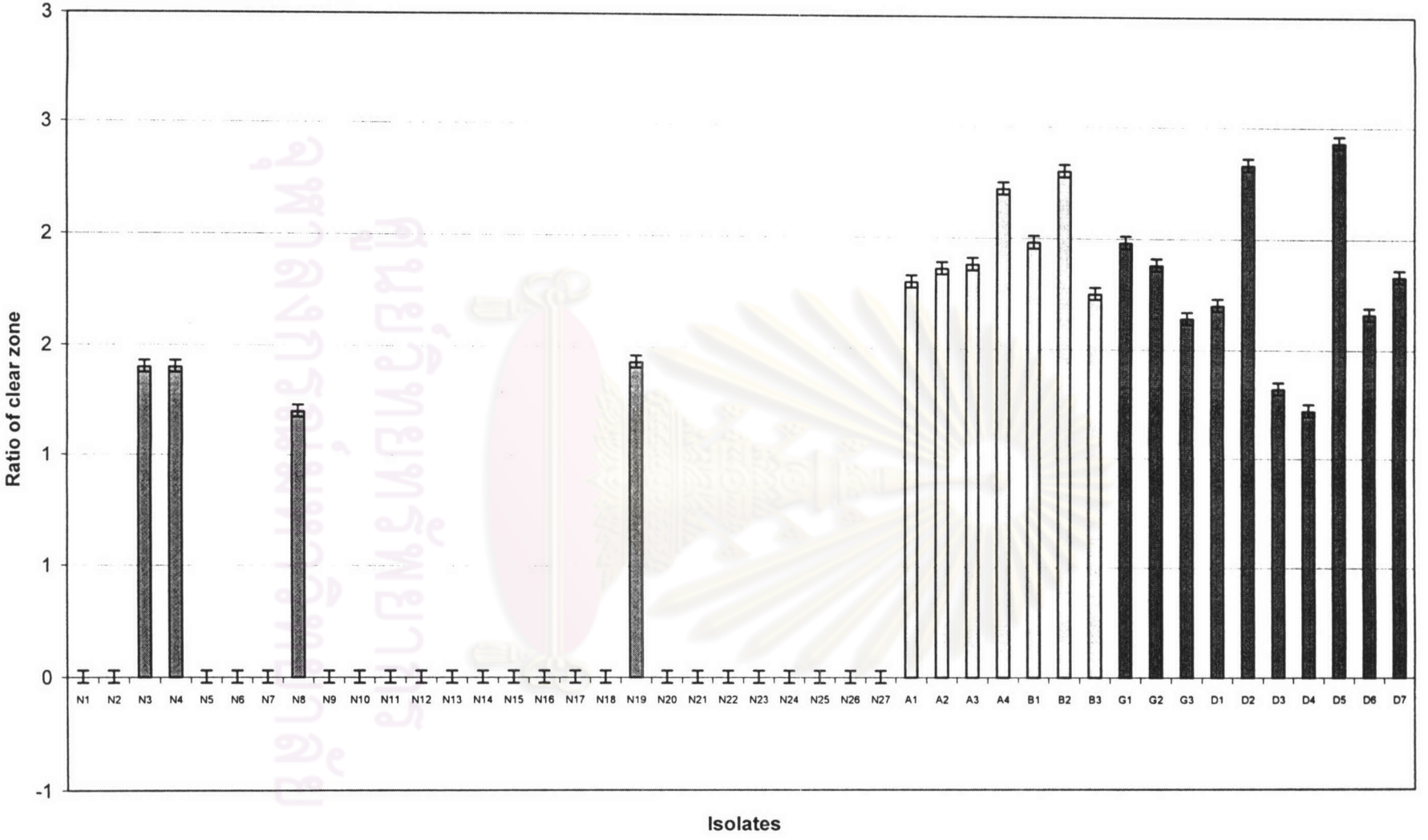

Figure 4.4 The ratio of clear zones of bacteria isolated from various sources after growing on skim milk agar plates for 1 day : bacteria from N. Maneechote (N1-N27), decomposed vegetables (A1-A4, B1-B3), soil around the food waste dumping area (G1-G3) and soil from the hot spring area (D1 – D7).

From the results, the bacteria from decomposed vegetables (A4 and B2) and bacteria from soil around hot spring area, Sankampang, Chiang Mai, Thailand (D2 and D5) provided the widest clear zones, 2.22, 2.30, 2.33 and 2.43 respectively. Therefore, these 4 isolates were selected for the further protease assay.

4.1.3 Isolation of lipase producing bacteria

Figure 4.5 showed the bacteria that could fluoresce. From the results, 2 isolates of *Bacillus subtilis,* 1 isolate of denitrifying bacteria and 3 isolates of bacteria from the soil around food waste dumping area were found to fluoresce (Table 4.1) and the qualitative fluorescence obtained was compared through the observations .These bacteria were selected for the further lipase assay.

Table 4.1 The fluoresced colonies of bacteria isolated from various sources: bacteria from Maneechote, 2001, soil around the food waste dumping area and denitrifying bacteria (Smartivutikoon, 2003).

Isolate(s)	Sources of bacteria	Fluorescent colonies*	Isolate(s)	Sources of bacteria	Fluorescent colonies*
N1	Maneechote (2001)	-	J1	Denitrifying bacteria, Smartivutikoon (2003)	-
N2		-	J2		-
N3		-	J3		-
N4		-	J4		-
N5		-	J5		-
N6		+	J6		-
N7		-	J7		-
N8		-	J8		-
N9		-	J9		-
N10		-	J10		++
N11		-	J11		-
N12		-	J12		-
N13		-	J13		-
N14		-	J14		-
N15		-	J15		-
N16		-	J16		-
N17		-	A1	Decomposed vegetables 1	-
N18		-	A2		-
N19		-	A3		-
N20		-	A4		-
N21		-	B1	Decomposed vegetables 2	-
N22		-	B2		-
N23		-	B3		-
N24		-	G1	Soil around food waste dumping area	++
N25		-	G2		++
N26		++	G3		++
N27		-			

* +, ++ referred to the degree of the fluorescence production on Rhodamine B agar plates.

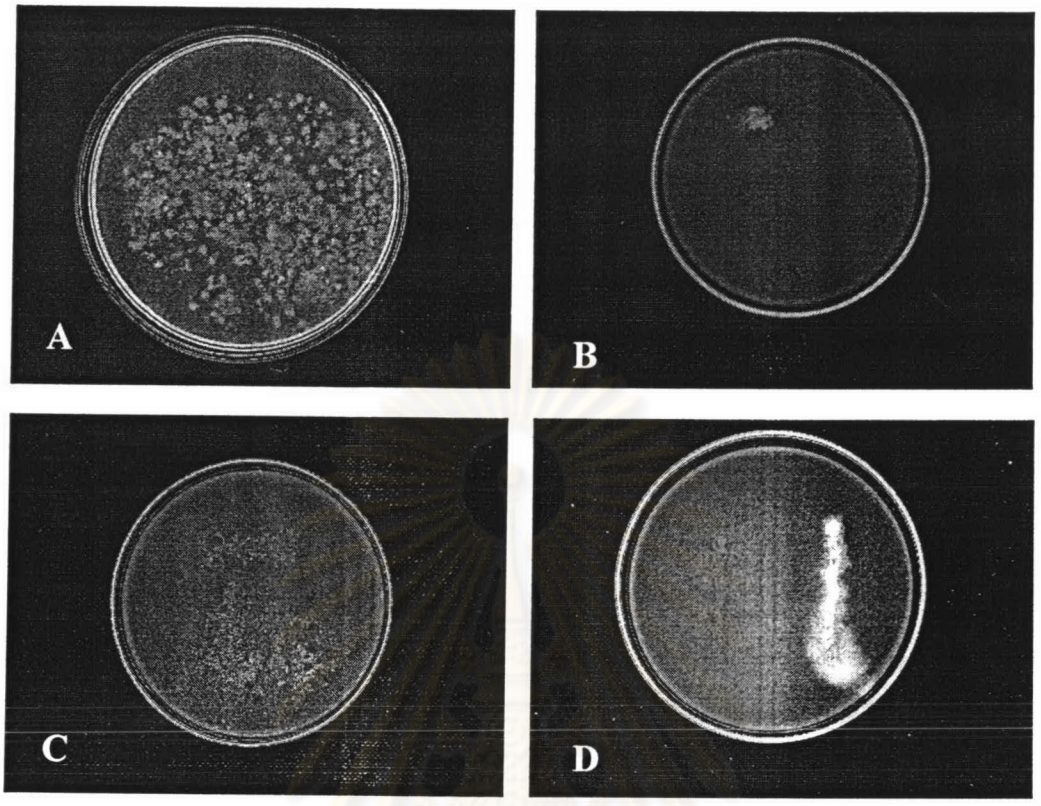

Figure 4.5 The fluorescence production on Rhodamine B agar plates from bacteria: A) serial dilution of sample from soil around food waste dumping area; B) *B. subtilis* N26; C) bacteria from food waste dumping; D) denitrifying bacteria (J10).

4.2 Assay of enzyme activities

4.2.1 Cellulase activity

When 5 isolates with the highest ratio of clear zones (P1, P2, P3, Y1 and Y2) were measured for cellulase activities, the results were shown in Table 4.2.

Table 4.2 Cellulase activities of selected bacteria.

Isolates	CMCase activity (U / ml)	Protein concentration (mg/ml)	Specific activity (U/mg protein)
P1	54.05	0.76	71.12
P2	33.89	0.78	43.72
P3	27.03	0.71	38.02
Y1	49.85	0.73	68.56
Y2	49.06	0.62	78.88

From the result (Table 4.2), 4 isolates of bacteria that produced the highest CMCase activity. P1, P2, Y1 and Y2 were selected for subsequent biofertilizer production.

4.2.2 Protease activity

Eighteen isolates of bacteria which produced clear zones on skim milk agar were selected for the protease activity measurement. The results were shown in Table 4.3.

Table 4.3 Protease activities of bacteria

Isolates	Protease activity (U / ml)	Protein concentration (mg/ml)	Specific activity (U/mg protein)
A1	4.73	0.21	11.10
A2	4.40	0.23	9.73
A3	6.67	0.23	14.76
A4	11.33	0.22	25.29
B1	5.80	0.21	13.88
B2	11.09	0.26	21.49
B3	4.80	0.27	8.99
N3	0.85	0.28	1.52
N4	2.20	0.29	3.75
N8	1.07	0.26	2.10
N19	0.80	0.26	1.56
D1	4.38	0.30	7.37
D2	11.24	0.31	17.90
D3	4.66	0.27	8.60
D4	4.96	0.29	8.58
D5	13.73	0.19	36.32
D6	5.24	0.23	11.54
D7	1.33	0.26	2.52

From the results, 4 isolates of bacteria (A4, B2, D2 and D5) which provided the highest protease activities, were therefore selected for biofertilizer production.

4.2.3 Lipase activity

Six isolates of fluorescence producing bacteria were selected for the lipase assay. The results were shown in Table 4.4

Table 4.4 Lipase activities of selected bacteria

Isolate	Lipase activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg protein)
G1	2.75	6.07	0.23
G2	2.38	5.62	0.21
G3	1.25	6.03	0.12
N6	0.50	6.81	0.04
N26	2.25	5.10	0.22
J10	1.50	6.14	0.15

From the results, 4 isolates, G1, G2, N26 and J10 were later used for biofertilizer production.

4.3 Identification of bacteria

4.3.1 Gram's stain

When 12 isolates of bacteria (P1, P2, Y1, Y2, A4, B2, D2, D5, G1, G2, N26 and J10) were stained, the results revealed that 10 isolates of bacteria were gram positive and rod shape, 1 isolate (J10) was gram negative bacilli and the other (Y2) was gram negative rod (Figure 4.6, 4.7, 4.8). The morphology of bacterial colonies growing on nutrient agar plates were also observed (Table 4.5).

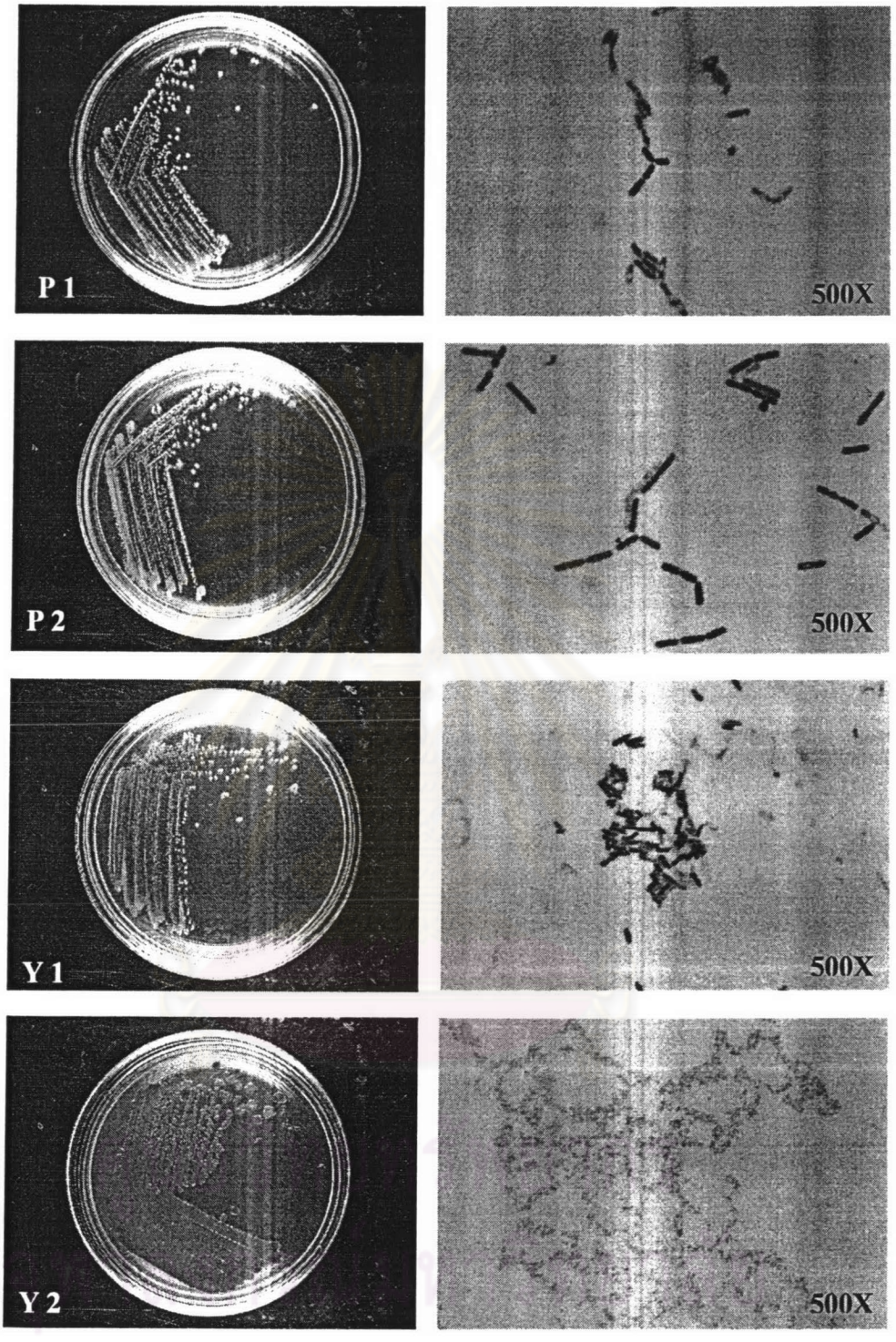

Figure 4.6 Morphology of the colonies and gram's stain of selected cellulase-producing bacteria growing on nutrient agar plates at room temperature (28°C) for 1 day: The right row illustrates the 500 times magnification of the colonies.

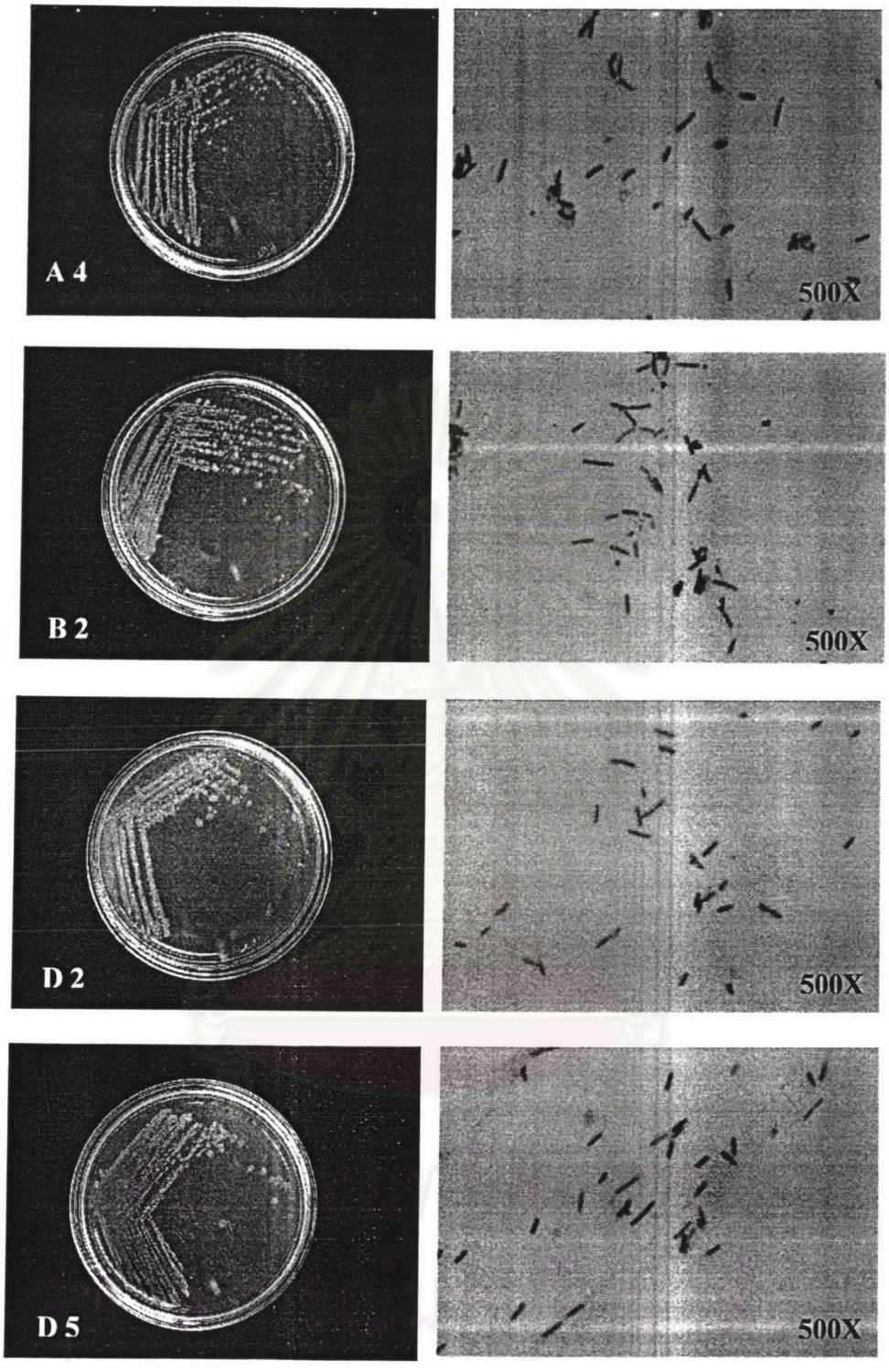

Figure 4.7 Morphology of the colonies and gram's stain of selected protease-producing bacteria growing on nutrient agar plates at room temperature (28°C) for 1 day: The right row illustrates the 500 times magnification of the colonies.

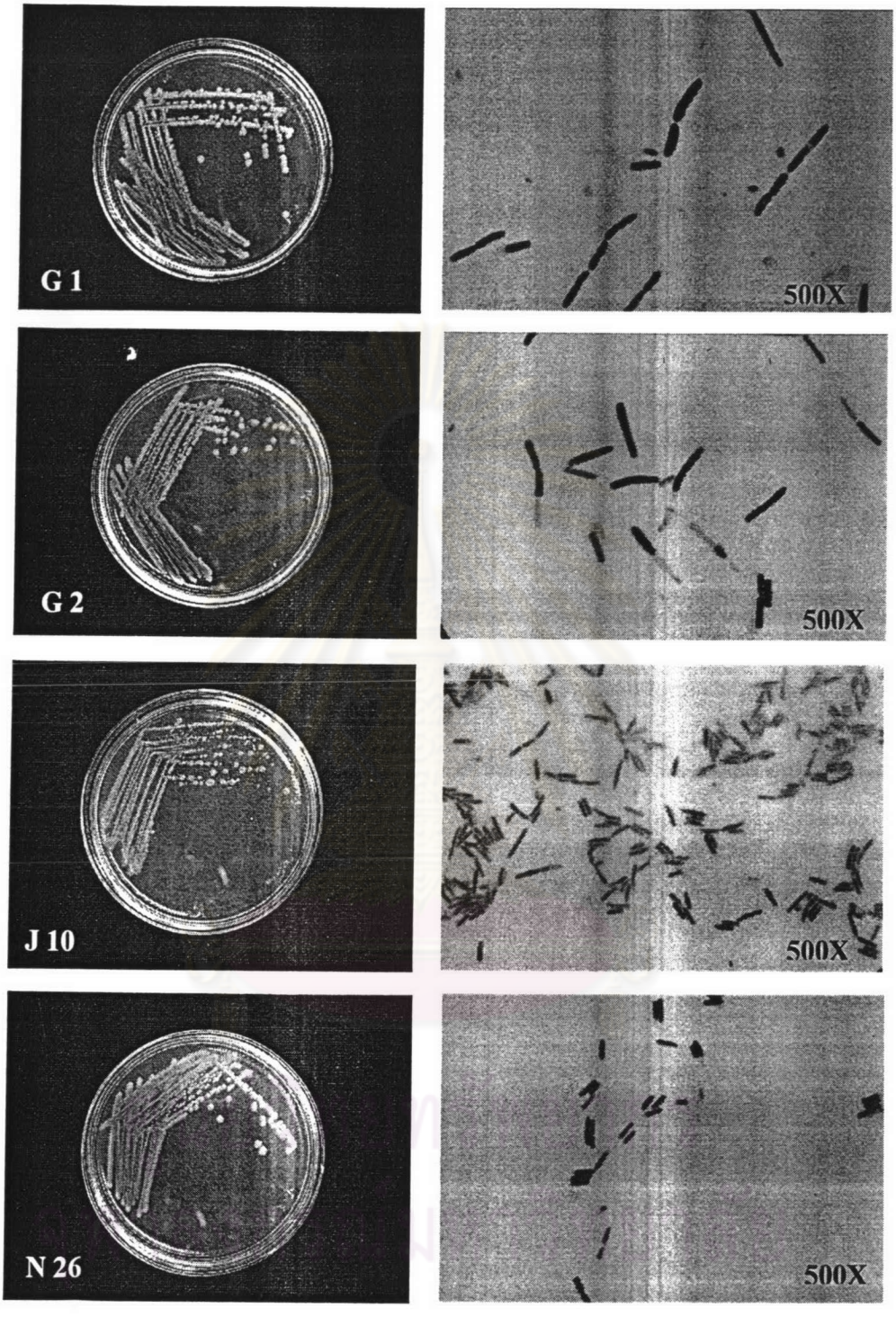

Figure 4.8 Morphology of the colonies and gram's stain of selected lipase-producing bacteria growing on nutrient agar plates at room temperature (28°C) for 1 day: The right row illustrates the 500 times magnification of the colonies.

4.3.2 Oxidase test

When the selected colonies were streaked on the filter paper soaked with 1% TMPD to observe the purple color of the colonies along the streaked line, 12 isolates gave positive results shown in Table 4.5.

4.3.3 Catalase test

The O_2 production from the bacteria in 3% hydrogen peroxide was shown in Table 4.5.

4.3.4 Biochemical test

The results of biochemical test of the selected bacteria were shown in Table 4.6 and 4.7.

Table 4.5 Morphology of the colonies, Gram's stain, Oxidase test, Catalase test and name of the selected bacteria.

Bacterial isolates	Name of bacteria	Morphology of the colonies on nutrient agar plates	Gram's stain	Cell shape	Oxidase test*	Catalase test**
P1	*Bacillus cereus*	Light yellow, smooth margin	Positive	Rod	+	+
P2	*Bacillus cereus*	Light yellow, smooth margin	Positive	Rod	+	+
Y1	*Bacillus subtilis*	Light yellow, smooth margin	Positive	Rod	+	+
Y2	*Serratia marcescens*	Red colonies, smooth margin	Negative	Rod	+	++
A4	*Bacillus coagulans*	Light yellow, smooth margin	Positive	Rod	+	+
B2	*Bacillus coagulans*	Light yellow, smooth margin	Positive	Rod	+	+
D2	*Bacillus coagulans*	Light yellow, smooth margin	Positive	Rod	+	+
D6	*Bacillus coagulans*	Light yellow, smooth margin	Positive	Rod	+	+
G1	*Bacillus cereus*	Light yellow, smooth margin	Positive	Rod	+	++
G2	*Bacillus cereus*	Light yellow, smooth margin	Positive	Rod	+	++
N26	*Bacillus cereus*	Light yellow, smooth margin	Positive	Rod	+	+
J10	*Pseudomonas aeruginosa*	Light yellow, smooth margin	Negative	Bacilli	+	+

* + refer to positive result of oxidase test,

** +, ++ refer to quantity of gas production from catalase test.

Table 4.6 Biochemical test of six isolates of the selected bacteria.

Biochemical test	P1	P2	Y1	Y2	A4	B2
TSI/H_2S	K/A/-	K/A/-	K/A/-	K/A/-	K/A/-	K/N/-
Indole/motile	nt*	nt	nt	-/+	nt	nt
Simmon's citrate	+	+	+	nt	+	+
Urease	+	+	-	-	+	+
Nitrate/N2 gas	+/-	+/-	+/-	nt	+/-	+/-
Esculin	+	+	+	+	+	+
VP	+	+	-	+	-	-
Gelatinase	+	+	+		+	+
Glucose	+	+	+	+	+	+
Maltose	nt	nt	nt	+	nt	nt
Lactose	nt	nt	nt	-	nt	nt
Mannitol	-	-	+	+	+	+
D-Xylose	-	-	+	-	+	+
Rhamnose	-	-	-	-	-	-
Sucrose	nt	nt	nt	+	nt	nt
Adonitol	nt	nt	nt	+	nt	nt
L-Arabinose	-	-	+	-	+w	-
Salicin	-	+w	-	+	-	-
Lysine decarboxylase	nt	nt	nt	+	nt	nt
Arginine dihydrolase	+	+	-	-	-	-
Ornithine decarboxylase	nt	nt	nt	+	nt	nt
Egg yolk	+	+	-	nt	-	-
Result	*Bacillus cereus*	*Bacillus cereus*	*Bacillus subtilis*	*Serratia marcescens*	*Bacillus coagulans*	*Bacillus coagulans*

* nt = no testing.

Table 4.7 Biochemical test of six isolates of the selected bacteria.

Biochemical test	D2	D5	G1	G2	N26	J10
TSI/H_2S	K/A/-	K/A/-	K/A/-	K/A/-	K/A/-	K/N/-
Indole/motile	nt*	nt	nt	nt	nt	-/+
Simmon's citrate	+	+	+	+	+	+
Urease	+	+	+	+	+	+
Nitrate/N2 gas	+/-	+/-	+/-	+/-	+/-	+/+
Esculin	+	+	+	+	+	-
Acetate	nt	nt	nt	nt	nt	+
VP	-	-	+	+	+	
Gelatinase	+	+	+	+	+	-
Glucose	+	+	+	+	+	+
Maltose	nt	nt	nt	nt	nt	-
Lactose	nt	nt	nt	nt	nt	-
Mannitol	+	+	-	-	-	+
D-Xylose	+	+	-	-	-	+
Rhamnose	-	-	-	-	+w	
Sucrose	nt	nt	nt	nt	nt	-
Adonitol	nt	nt	nt	nt	nt	-
L-Arabinose	-	+w	-	-	-	
Fructose	nt	nt	nt	nt	nt	+
Salicin	-	-	+w	+w	+w	
Lysine decarboxylase	nt	nt	nt	nt	nt	-
Arginine dihydrolase	-	-	-	-	-	+
Ornithine decarboxylase	nt	nt	nt	nt	nt	-
Egg yolk	-	-	+	+	+	nt
Result	*Bacillus coagulans*	*Bacillus coagulans*	*Bacillus cereus*	*Bacillus cereus*	*Bacillus cereus*	*Pseudomonas aeruginosa*

* nt = no testing.

From Table 4.5, 4.6 and 4.7, the isolates from the compost from The Royal Project were *Bacillus cereus, Bacillus subtilis* and *Serratia marcescens.* The isolates from decomposed vegetables and soil from hot spring area were *Bacillus coagulans.* The isolate from soil around food waste dumping area was *Bacillus cereus* and the denitrifying bacterium provided by K. Smartivutikoon was *Pseudomonas aeruginosa.* Interestingly, the bacterial isolate N26, previously identified as *Bacillus subtilis* (Maneechote, 2001) was re-identified as *Bacillus cereus* in this study.

4.4 Antagonistic test of the selected bacteria

The selected bacteria were cross-streaked across each other to test for antagonistic effect on growth on the nutrient agar plates. All bacterial isolates could grow together on the same plate as shown in Figure 4.9. Since antagonistic effect could not be detected, all selected bacteria could be used for biofertilizer production in the same pot.

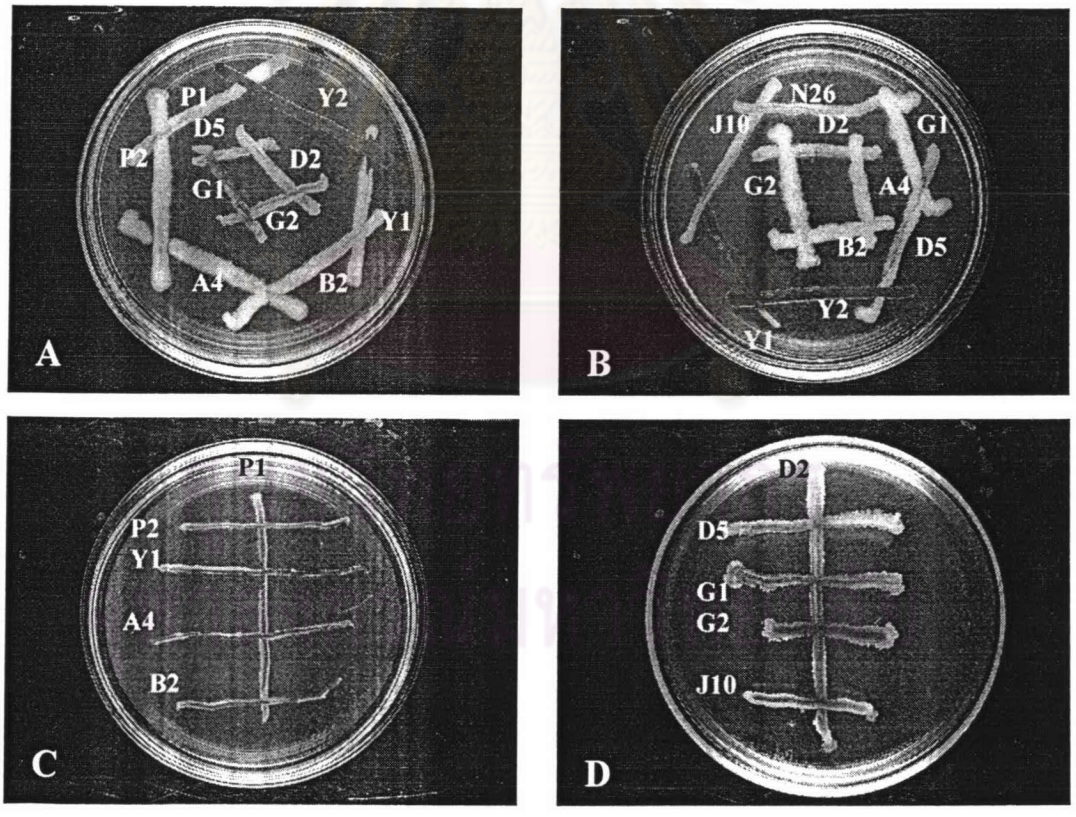

Figure 4.9 Antagonistic test of the selected bacteria: A and B) 10 isolates of bacteria grown together on nutrient agar plate; C and D) 5 isolates of bacteria grown together on nutrient agar plate.

4.5 Biofertilizer production

4.5.1 Temperature and pH of the biofertilizer

Six pots of liquid biofertilizer were left stand for 30 days and the results were depicted in Figure 4.10.

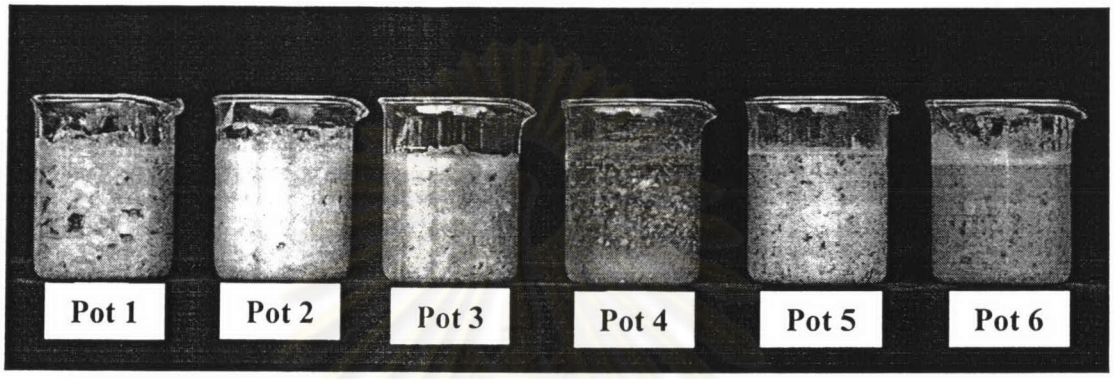

Figure 4.10 The 30 day old biofertilizer: Pot 1) home food waste; Pot 2) home food waste inoculated with mixed bacteria; Pot 3) synthetic waste, Pot 4) synthetic waste inoculated with mixed bacteria; Pot 5) autoclaved synthetic waste; Pot 6) autoclaved synthetic waste inoculated with mixed bacteria.

The temperature was measured every 3 days and the pH was measured every 5 days of composting. The moisture content in each pot was measured at the beginning and the end of composting. The results were shown in Figure 4.11, 4.12 and Table 4.8, respectively.

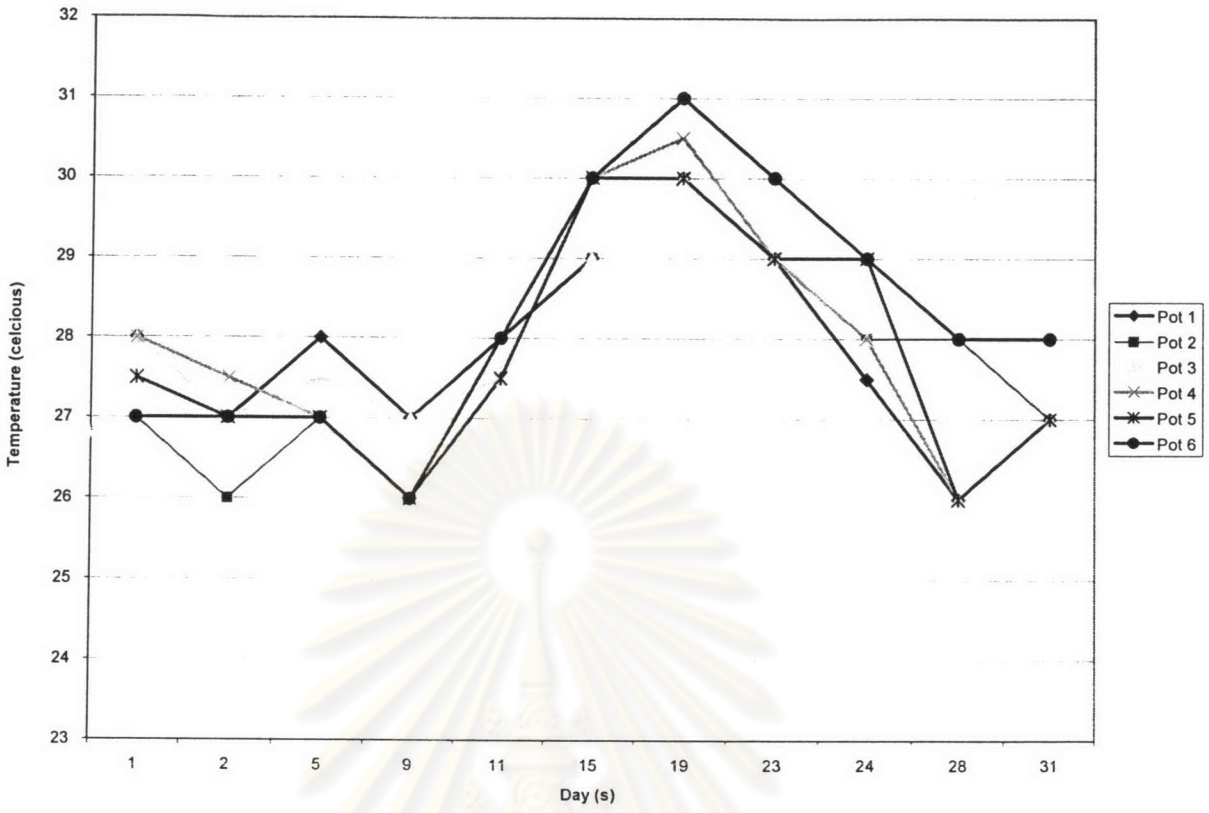

Figure 4.11 Temperature of the biofertilizer was measured every 3 days of composting: Pot 1) home food waste; Pot 2) home food waste inoculated with mixed bacteria; Pot 3) synthetic waste, Pot 4) synthetic waste inoculated with mixed bacteria; Pot 5) autoclaved synthetic waste; Pot 6) autoclaved synthetic waste inoculated with mixed bacteria.

From the results in Figure 4.11, the temperature in the biofertilizer pots was increased to about 30 – 31°C during the time of composting and decreased to room temperature (27-28°C) at the end of composting.

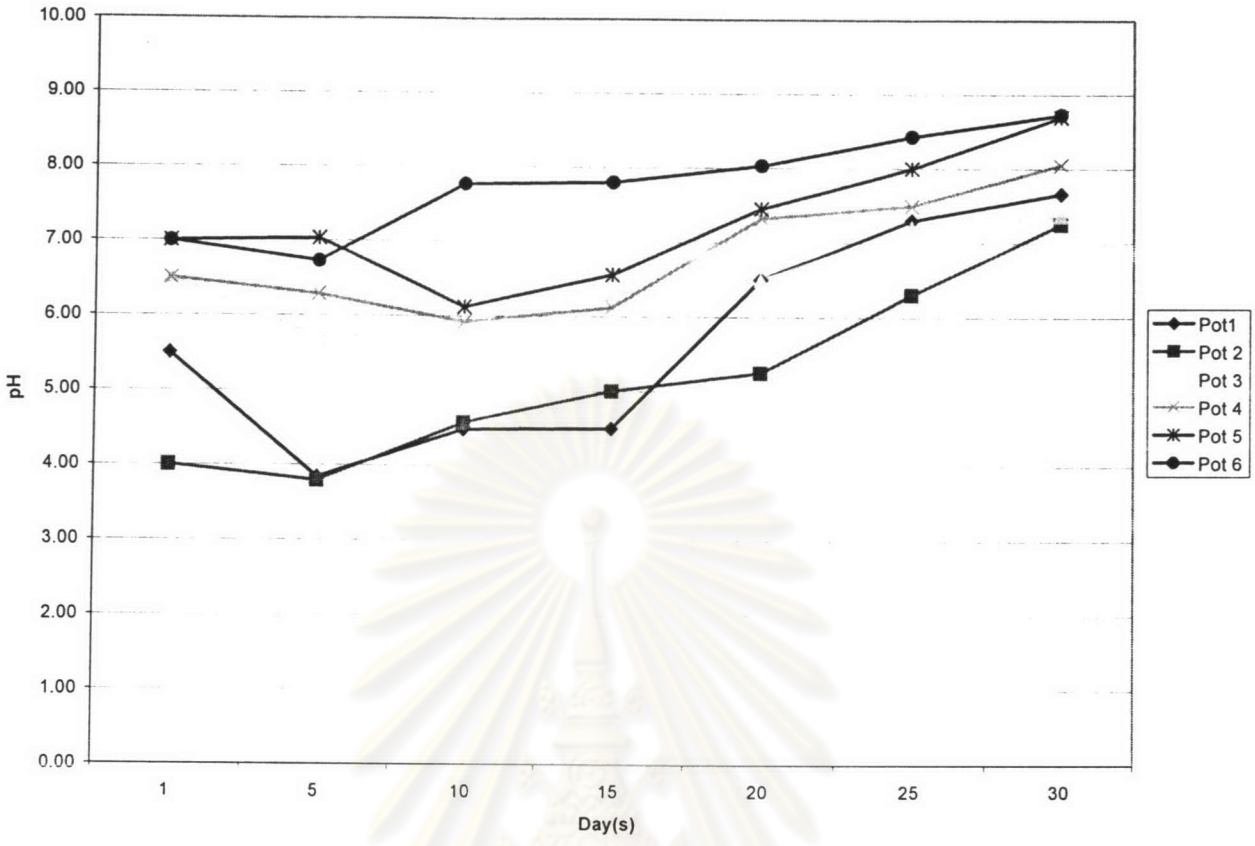

Figure 4.12 The pH of the biofertilizer in the pot was measured every 5 days of composting: Pot 1) home food waste; Pot 2) home food waste inoculated with mixed bacteria; Pot 3) synthetic waste. Pot 4) synthetic waste inoculated with mixed bacteria; Pot 5) autoclaved synthetic waste; Pot 6) autoclaved synthetic waste inoculated with mixed bacteria.

From Figure 4.12, pH of the biofertilizer was ranged from slightly acidic to neutral (pH 4-7) at the beginning of composting and increased to basic (pH 7-8.5) at the end of composting.

4.5.2 Moisture content and nutritional values

At the beginning and the end of composting, the moisture contents were measured and the results were shown in Table 4.8.

Table 4.8 Moisture content of the biofertilizer

Pot number	Moisture content (%)	
	Day 0	Day 30
1	70.67	81.73
2	73.25	82.60
3	72.26	82.13
4	75.51	81.76
5	70.31	83.70
6	74.85	82.18

From Table 4.8, it was shown that the biofertilizers were relatively damp and more moisture content was detected at the end of composting than at the beginning. Moreover, the waste texture were decomposed to semi-solid state as shown in Figure 4.10.

4.5.3 Carbon and nitrogen content

When the biofertilizer was measured for carbon and nitrogen contents, the results were shown in Table 4.9.

Table 4.9 Carbon and nitrogen ratio of the biofertilizer.

Pot number	C:N ratio		Decreasing percent of C:N ratio
	Day 0	Day 30	
1	19.61	16.80	14.33
2	19.07	13.08	31.41
3	19.65	13.08	33.44
4	19.26	15.74	18.28
5	19.36	8.00	58.68
6	18.63	7.53	59.58

From Table 4.9, the C:N ratios before composting of all 6 pots were in normal range value of 20:1. As time progressed through the end of composting, the C:N ratios in pot 5 and 6 were dramatically decreased as much as 58.68 and 59.58%, respectively, while the other 4 pots showed relatively similar changes, 14.33, 31.41, 33.44 and 18.28% decrease in C:N ratio.

The nutritional values of the biofertilizers, nitrogen, phosphorus and potassium, were measured after 30 days of composting. The results were shown in Table 4.10.

Table 4.10 Nutritional values of the biofertilizer.

Pot number	Total Nitrogen (%)	Total Phosphorus (%)	Total Potassium (%)
1	1.10	1.08	1.18
2	1.31	1.10	1.21
3	1.30	1.09	1.23
4	1.40	1.13	1.27
5	1.36	1.16	1.20
6	1.42	1.20	1.23

From the results in Table 4.10, it could be concluded that the nutritional values in the biofertilizer were not significantly different among all groups of fertilizers.

4.6 Efficiency of the liquid biofertilizer

4.6.1 Plant growth

The experiment was designed by randomized block design (RBD) with 8 replicates. The biofertilizers were provided to the plant after one week of transplantation. The biofertilizers were diluted in the ratio of 1:500 (recommended by Land Development Department). The experiments consisted of 7 treatments.

All diluted biofertilizers were poured to the plant every morning for 150 ml. Plant heights were measured every 3 days. After 30 days of planting, all plants were harvested. Plant fresh weight was measured after harvesting. The data were analyzed by ANOVA using program SPSS version 10.0. The appearance of the plants was shown in Figure 4.13. Plant height and fresh weights were shown in Figure 4.14 and 4.15.

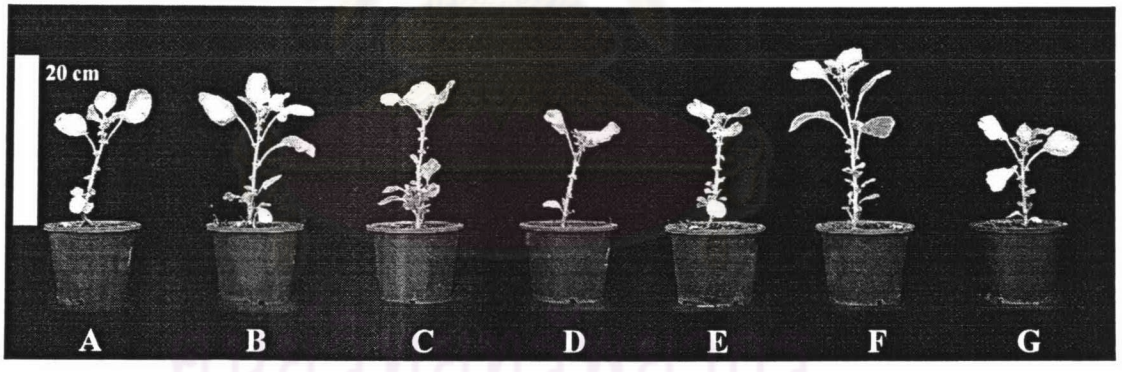

Figure 4.13 Growth of 30 day old Chinese spinach (*Amaranthus viridis* L.). Plants were grown in 6-inch diameter pots containing 500 grams of soil and the tested plants were provided with: A) biofertilizer from home food waste; B) biofertilizer from home food waste inoculated with mixed bacteria; C) biofertilizer from synthetic waste, D) biofertilizer from synthetic waste inoculated with mixed bacteria; E) biofertilizer from autoclaved synthetic waste; F) biofertilizer from autoclaved synthetic waste inoculated with mixed bacteria; G) water in control group.

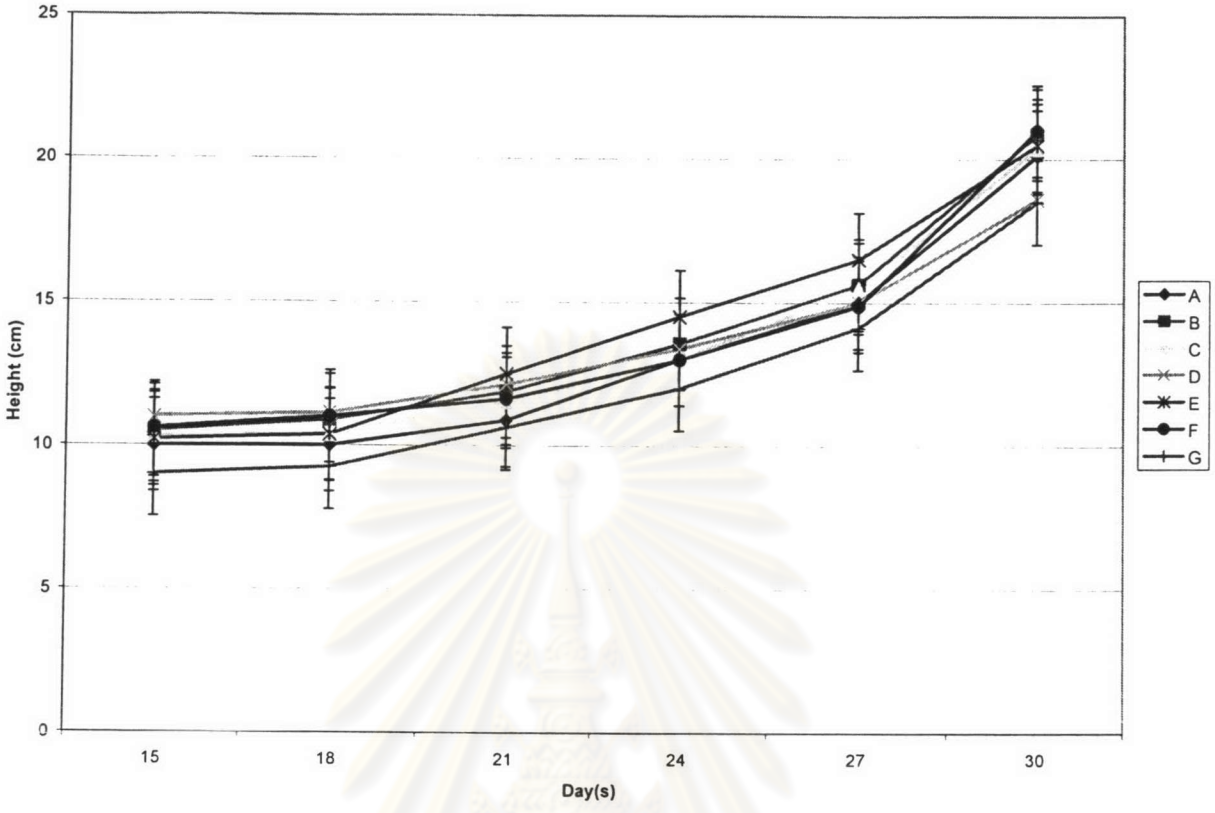

Figure 4.14 Height of Chinese spinach (*Amaranthus viridis* L.). Plants were grown in 6-inch diameter pots containing 500 grams of soil and the tested plants were provided with: A) biofertilizer from home food waste; B) biofertilizer from home food waste inoculated with mixed bacteria; C) biofertilizer from synthetic waste, D biofertilizer from synthetic waste inoculated with mixed bacteria; E) biofertilizer from autoclaved synthetic waste; F) biofertilizer from autoclaved synthetic waste inoculated with mixed bacteria; G) water in control group.

From Figure 4.14, the Chinese spinach treated with biofertilizer of autoclaved waste with mixed bacteria was the tallest 21.00 cm. Plants received biofertilizer of home food waste with mixed bacteria, biofertilizer of autoclaved waste, biofertilizer of synthetic waste, biofertilizer of home food waste and biofertilizer of synthetic waste inoculated with mixed bacteria were also higher when compared to the control. However, there was no significant difference among all treatments.

Plant fresh weights were measured after 30 days of planting. All part of the plants, including shoot and root were harvested, cleaned and measured. The results were shown in Figure 4.15.

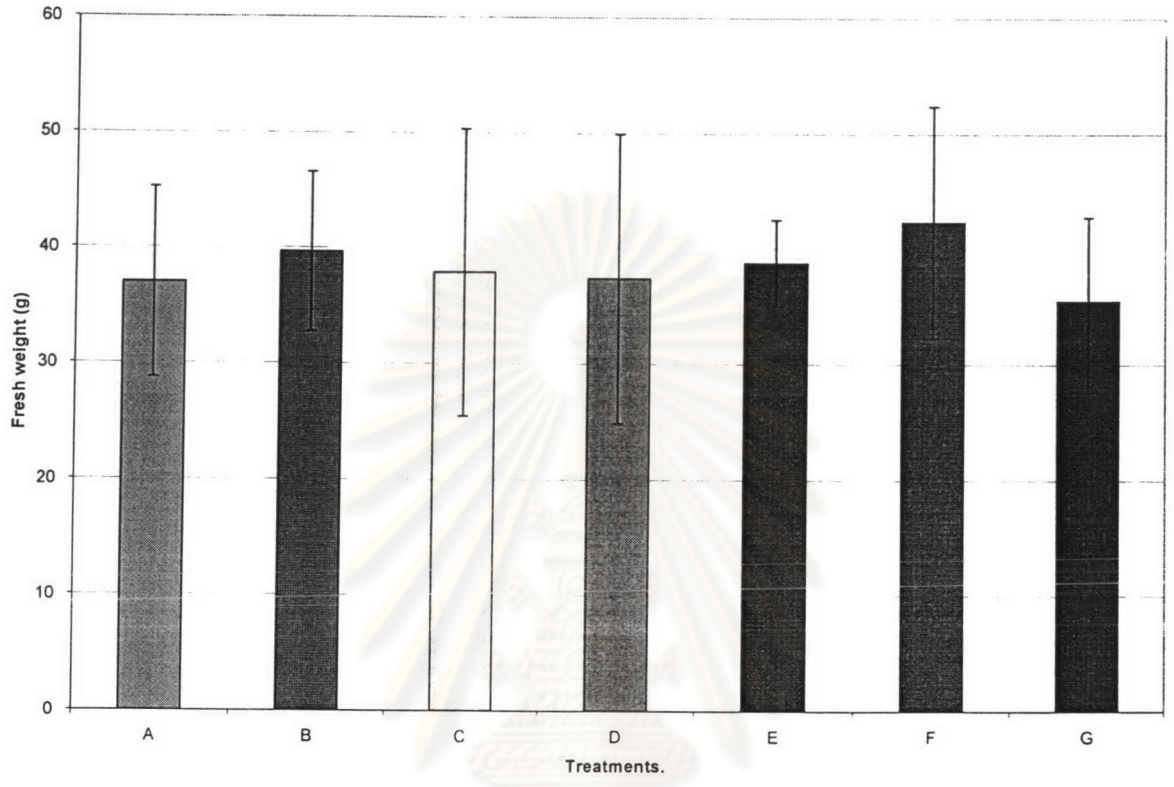

Figure 4.15 Plant fresh weight after 30 days of planting: A) received the biofertilizer from home food waste; B) received the biofertilizer from home food waste inoculated with mixed bacteria; C) received the biofertilizer from synthetic waste, D) received the biofertilizer from synthetic waste inoculated with mixed bacteria; E) received the biofertilizer from autoclaved synthetic waste; F) received the biofertilizer from autoclaved synthetic waste inoculated with mixed bacteria; G) control group.

From the results, the plants received the diluted liquid biofertilizer of autoclaved synthetic waste inoculated with the selected bacteria (F) had the highest fresh weight, 42.33 g. while the rest of the plants treated with other types of liquid biofertilizer showed lower values, 39.66 (B), 38.78 (E), 37.91 (C), 37.39 (D) and 37.01 (A), respectively. The control plants (with no biofertilizer) showed the lowest fresh weight (35.50 g). Obviously, the results obtained from the fresh weight were consistent with the height.