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IDENTIFICATION OF SOME SELECTIVE ISOLATES OF THERMOPHILIC ACTINOMYCETES DURING STORAGE OF FARM YARD MANURE.

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ABSTRACT

A heap of farm yard manure (2 x 2 x 1.5 m height) was stored for l00 days. Periodical samples were collected from the heap after initial, 2, 4, 6, 8, 10 and 12 weeks for isolation of some thermophilic actinomycetes, and determination of their enzymatic activities to decompose cellulose by the determination of C_1 & C_x cellulases. The counts of total bacteria, spore formers, aerobic cellulose decomposing bacteria, fungi, mesophilic actinomycetes and thermophilic actinomycetes were carried out. The recorded temperature of the heap at the depth of 60-40 cm was 70ºC after 4 weeks from the beginning of the storage. The pH showed continuous decreases from 8.2 to 6.5 at the end of the experiment.

The microbiological studies of the collected samples showed higher counts of total bacteria then decreased till the end of the experiment. The spore formers reached the highest counts after 4 – 5 weeks then decreased. The counts of aerobic cellulase decomposing bacteria reached maximum after 6 weeks then decreased to the end of the experiment, as well as the counts of fungi has the same pattern. The mesophilic actinomycetes showed higher counts at the beginning of the storage then decreased on the expense of the thermophilic actinomycetes, which greatly increased by increasing of heap temperature; reached maximum after 4 – 6 weeks; while the mesophiles diminished.

Sixty isolates of thermophilic actinomycetes were isolated from the heap for purification and identification these isolates were identified according to Bergey's manual (1998) were related to 5 species of them 3 genera which were highly active namely. Thermoactinomyces, Thermomonospora and streptomyces respectively. The enzymatic activity of these strains showed high activity of C_1 and Cx cellulases. The highest active Thermoactinomyces saccharia, thermoactinomyces *vulgaris, Thermomonospora fusca*, *Thermomospora alba and Streptomyces thermofuscus*.

INTRODUCTION

Composting is a general treatment method for agricultural wastes recycling. Although several reports are available concerning the composition and dynamics of the microflora during composting of these wastes. (Mc-kinely and Vestal, 1984). Little is known about the microbial diversity during the composting of the organic fraction of these wastes. Although, general tendencies with regards to mesophilic and thermophilic population of microorganisms have been identified during composting (Fym) of these wastes knowledge of the importance of specific taxonomic and functional groups can still be improved Bergy's manual (1998).

Furthermore, monitoring of the microbial succession is important in effective management of the composting (FYM) process as microorganisms play roles in the process and the appearance of some microorganisms reflects the quality of maturing compost (FYM) (Bess, 1999). Comprehensive

study of thermophilic streptomycetes was carried out by Good Fellow *et al.,* (1987). They compared 50 thermophilic streptomyces from diverse habitats with representative mesophilic neutrophilic marker strains had been included in the extensive numerical taxonomic survey of Wiliams et al., (1983).

Shahin, (1995), isolated large number of the thermphilic streptomycetes from soil adjusted to pH 7.2 and 10.5 at 55^{o}C for 5 days. One hundred and twenty-nine representative isolates of thermophilic streptomycetes have been received little attention to their possible importance in microbial technology.

The mesophilic actinomycetes showed gradual increase throughout the composting and decreased when compost was incubated at 60^{o}C. Thermophilic actinomycetes behaved lie bacteria but high counts persisted for longtime. Thermophilic actinomycetes can grow at higher temperature than thermophilic fungi; hence, actinomycetes become dominant at higher temperature phase of composting (Taha *et al.,* 1968). At the later stages of composting the numbers of Actinomycetes (mesophilic and thermophilic) radically decreased. The ratio of mesophilic to thermophilic actinomycetes count was less after the first 4 weeks and then increased in the next weeks of composting period (Godden and Peninch, 1984).

The present study aimed to constructing a more complete picture of the culturable microflora during small-seal composting bin of (FYM) from starting material to mature compost rate only with regard to obiotic factors but also, with regard to the microbial activity (bacteria and actinomycetes) and enzymatic activities evaluation of functional and taxonomic groups of actinomycetes which play major role in decomposition process according to their efficiency to produce cellulose decomposing (C_1 & C_x) enzymes.

MATERIALS AND METHODS

A heap of farmyard manure (2 x 2 1.5 m) was stored for 100 days. Periodical samples were collected from this heap after 0, 2, 4, 6, 8 10 and 12 weeks.

Temperature measurement:

Temperature was measured at ranged between depths (40 - 60 cm) around heap center using a thermo-couple thermometer.

ph value:

pH values were determined in (FYM) water mixtures (1 : 5) using a pH glass electrode or ion expandable ion analyzer EAg 20.

Microbiological analyses:

Total counts of microorganisms in (FYM):

The serial dilution plate count procedure was used to estimate the total number of different groups of microorganisms, namely, bacteria, spore-forming bacteria aerobic cellulose decomposing bacteria, fungi as will as mesophilic and thermophilic actinomycetes. The selective media were used

for plate counts. These were, nutrient agar medium (Difco, 1966) for count of bacteria and spore-forming bacteria counts; potato dextrose agar (P-D-A) (Booth, 1971) for total count fungi; Duboss cellulose medium (Allen, 1953) was used for aerobic cellulose decomposing bacteria; inorganic salts starch agar medium (Szabo, 1990) was used for mesophilic actinomycetes and Kosmatchev agar medium (Kosmatchev, 1964) was used for thermophilic actinomycets.

Isolation and purification of thermophilic actinomycetes:

Identification of the isolated strains of thermophilic actinomycetes up to seria and genera was carried out according to the diagnostic Key of Bergey's Manual, (1998).

Cultural characteristics:

All isolates were streaked on Kosmatcher agar medium plates. The inoculated agar plates were incubated at 55^{o}C for 10 days for thermophilic actinomycetes. The color of aerial and substrate mycelium, intensity of growth, pigment production and the growth characteristics were recorded as mentioned by (Bergy's Manual, 1998).

Aerobic cellulose decomposing bacteria:

The dilution method was used. The most probable numbers of bacteria were obtained from the positive tubes using Hoskins (1934).

Production of C_1 and C_x Cellulases:

The production of C_1 Cellulase, Erlenmeyer flasks of 2 L capacity containing 400 ml of minimal medium (pH 7.2 – 7.4) and 2.0 g of ground filter paper were inoculated with 10.0 ml of spore suspensions each of the different thermophilic actinomycetes strains. Flasks were incubated for 3 weeks. The production of C_x- cellulose, 2 L capacity Erlenmeyer flasks, containing 400 ml of minimal medium (pH 7.2 – 7.4) and 2.0 g of carboxy methyl cellulose were inoculated with 10.0 ml spore suspensions of the different thermophilic actinomycetes strains. Flasks were incubated for 3 weeks at 55^{o}C. At the end of the incubation period, 1, 2 and 3 weeks the cultures were filtered by using whatman No 44 filter paper and sterilized by passing through seitz filter. The filtrates were used immediately for cellulases assays as follows. This was carried out to determine the activity of C_1-cellulase components; the enzyme which is necessary for hydrolysis of resistant substrates. The activity of C_x cellulase components; the enzyme which hydrolysis activated cotton and soluble cellulose derivative was assayed by the hydrolysis of carboxymethyl cellulose.

Assays for C_1 and C_x – Cellulase enzymes:

C_1 and C_x activities were determined by measuring the released reducing sugars by Mandels and Weber (1969).

RESULTS AND DISCUSSION

Table (1) showed the microbiological changes during storage of farm yard manure (FYM). Mean counts of bacteria were found to be order 310, 450, 220, 190, 175, 120 and 150 x 10^6 cfu/gm dry wt. after initial, 2, 4, 6, 8, 10 and 12 weeks. Bess, (1999), obtained the showed marked increases during the two weeks of (FYM) storage similar results. Mean total spore-forming bacteria were found to be 25, 90, 125, 170, 100, 75 and 45x10^6/g dry weight after initial, 2, 4, 6, 8, 10 and 12 weeks followed by marked decrease after 8 weeks. The aerobic cellulose decomposing bacteria showed a marked decrease during storage period namely 8 weeks.

This results agree with that obtained by Taha et al.(1968), and Cheng et al. (1988) who showed that a reduction in the population of cellulose decomposers during the high temperature phase followed by a rise due to the subsequent lower temperatures.

Mean counts of fungi showed marked increase during the 4 weeks period storage. Similar results were obtained by Kane and Mallanes. (1973), Griffin (1985). Mean counts of mesophilic actinomycetes showed marked increase during storage (FYM) the first two weeks, however, showed a drastic reduction during initial, 2, 4, 6, 8, 10 and 12 weeks. Mean counts of thermophilic actinomycetes of the composted (FYM) materials showed a marked increase during the high temperature (70 – 62^{o}C) with a significant peak after 4 and 6 weeks. The increase in the count of thermophilic actinomycetes during the early stages of (FYM) composting was in agreement with findings obtained by Limtong (1990), Hanafy et al., (1990), Beffa et al (1996) Kukalya (1996), Agarwal (1997), , and Nevzat, (2002).

Table (1): Total microbial counts during storage of farm yard manure (mean counts x 10^6/ cfu/gm dry wt.)

Time In Weeks	Microorganisms					
	Total Count of Bacteria	Spore-forming Bacteria	Cellulose decomposi on g bacteria	Count of fungi	Count of Actinomycetes	
					Thermophilic At 55^{o}C	Mesophilic At 30^{o}C
Initial	310	25	16	4	2	24
2	450	90	22	9	4	26
4	220	125	25	10	12	3
6	190	170	27	8	10	-
8	175	100	25	7	8	-
10	120	75	21	3	5	2
12	150	45	13	3	2	8

Temperature changes:

Temperature evaluation is an indicator of microbial during composting (FYM) process, consequently this parameter my considered as a good indicator.

The changing in temperature during period storage was daily recorded and showed table (2). Temperature of (FYM) composting process was measured at different depth (40 and 60 cm) from the center. The initial

stage storage up to 35°C has a very short duration of about 2 weeks. A progressive increase in temperature was measured at depth of 60 cm to stabilize above 70°C for 4 weeks. The encourage in the development of thermophilic microorganisms, mainly Eumycetes and Thermophilic actinomycetes was also recorded by Strom (1985) El-Gammal and Yousseri ((1980) and Kuhlman (1990) and Saleh (2003). The optimal range of temperature (40 – 60 °C) was enough for rapid and efficient decomposition and excellent compost production.

pH values:

Results in table (2) showed that pH value of the composting (FYM) materials of the heap was changed from 8.12, 8.8., 8.2, 7.7, 7.3, 6.9 and 6.5 after initial, 2, 4, 6, 8, 10 and 12 weeks, respectively. Decreasing in pH values may be due to the production of organic acids causing further acidification, during biooxidation phase.

Table (2): Changes in the temperature and pH values during storage period of (FYM)

Time in weeks	Temperature (˚C)	pH
Initial	35	8.12
2	40	8.8
4	70	8.2
6	62	7.7
8	60	7.3
10	52	6.9
12	38	6.5

Data in tables (3 and 4) showed the morphological and certain physiological characteristics of the thermophilic actinomycetes. All of the isolates grown at 55°C produced branched non fragmented vegetative mycelium bearing aerial hyphae. Single spores were formed on the branched sporophore. The pure isolates obtained were identified to genera and species according to the scheme presented by Bergey's Manual (1998). 29 isolates were not able to utilize arabinose, galactose and xylose, denoting that they are belonging to the genus thermoactinomyces. The percentage of this genus was found to be (48.3%). The twenty isolates of white group aerial mycelium were characterized by Growth at 55°C different degradation of starch with no formation of soluble pigments and utilization of carbon compounds. They were able to utilize sucrose. Hence, these isolates (33.3%) were identified as *Thermoactinomycetes sacchari.* The rest Nine isolates (15%) having with aerial mycelium. They don't produce soluble pigments, could utilized galactose and sucrose, with no growth on ribose and mannitol as a sole sources of carbon. Hence these isolates were identified as *Thermoactinomyces vulgaris.*

Twenty one isolates of the thermophilic actinomycetes produced branched nonfragmented vegetative. Mycellium bearing aerial hyphae and unbranched sporephore single spores, non sessile formed at the tip of sporephore are belonging to the genus, Thermomonospora. The rest thirteen isolates (21.7%) pale yellow aerial mycelium and could utilize galactose,

sucrose and mannitol with no ability to utilize ribose for growth. These isolates were identified as *Thermomonospora fusca.* The eight isolates white group were characterized by white substrate mycelium negative soluble pigments, degradation of starch and cellulose, and utilization of galactose and sucrose and not able to utilize ribose and mannitol were identified as *Thermomonospora albo (13.3 %)*

Ten isolates grown at 55°C having grey aerial mycelium, branched and bearing slightly spiral shaped spore chains were identified in the genus streptomyces.

The ten isolates grey groups were characterized by spiral spore chains, brown soluble pigment, degradation of the starch and not able to degrade cellulose and utilization of sucrose, galactose and mannitol and were not able to utilize ribose for growth. These isolates were identified as ***streptomyces thermofuscus*** (16.7%). These results are in agreement with Abo-Sedera (1995). Good fellow et al (1987) Kukaly et al., (1997) Albrecht ,Kampfer (2000) and White et al., (2003) who found that similar isolates of thermoactinomyces were good in all media containing.

Table (3): Identification to level genus of the thermoactinomyces isolated from a heap of storage farmyard manure at different periods

Growth at 55°C	Sporophore Morphology	Characters Of Spores	Growth carbon sources			Genus	Number Of isolates	
			Arabinose	Galactose	Xylose		Total	%
+	Branched	Single-sessile	-	-	-	Thermoactionomycetes	29	48.3
+	Un-branched		-	-	-	Thermomonospora	21	35
+	Spiral or Hooks and Open loop	Single-Non sessile Chain grey	-	+	-	Streptomyces	10	16.7

Table (4): Identification to level species of the thermophilic actinomycetes isolated from a heap of storage farmyard manure the at different periods.

Genus growth At 55° c	Colour of Aerial mycelium	Degradation of utilization of carbon compounds						Soluble pigment	Secies	No. of Sp.	
		Cellulose	Ribose	Mannitol	Galactose	Sucrose	Starch			No	%
Thermoactinomyces	White	-	+	+	+	-	+	-	*T. sacchari*	20	33.3
	White	-	-	-	+	+	-	-	*T-vulgaris*	9	15
Thermomonospora	Pale yellow	+	-	+	+	+	+	-	*T-fusca*	13	21.7
	White	+	-	-	+	+	+	-	*T-alba*	8	13.3
Streptomyces	Grey	-	-	+	+	+	+	Brown	*S.thermofuscus*	10	16.7

Screening for cellulytic ability of thermoactinomyces:

Results in table (5) showed that 31 out of 60 isolates were found to

be high cellulytic strains. Percentages of highly active strains to the total numbers of the same group were found to be 60%, 44.44%, 46.15%, 50% and 50% of total isolate of *Thermoactinomyces sacchari*, *Thermo-actinomyces vulgaris*, *Thermomonospora fusca*, *Thermomonospora alba* and *Streptomyces thermofuscus* respectively. These result are in agreement with Abd-El-Hafez et al (1971) Bond and Stutzenberger (1989) and Saleh, (2003). They showed that the thermophilic actinomycetes belonging to *Thermomonospora fusca* and *Thermomospora alba* were highly active cellulolytic organisms.

Table (5): The rate of cellulolytic activities of thermophilic actinomycetes isolated from the different storage of (FYM).

Thermophilic species	Total Of strains	Rate of Cellulolytic activities Number of isolates				% of highly active strains
		-	+	++	+++	
Thermoactinomyces sacchari	20	-	2	6	12	60
Thermoactinomyces vulgaris	9	3	-	2	4	44.44
Thermomonospora fusca	13	-	4	3	6	46.15
Thermomonospora alba	8	1	-	3	4	50
Streptomyces Thermofuscus	10	3	-	2	5	50
	60	7	6	16	31	51.66

-, Non cellulolytic; +, Low cellulolytic; ++, Medium cellulolytic; +++, High cellulolytic

Efficiency of cellulase activities:

The results of cellulase activity measured by C_1 and C_x produced by the most active strains of thermophilic actinomycetes isolated under this study. These results are shown in table (6). The obtained results showed a successive increase in cellulase activity of the most strains by increasing of incubation period from 1 – 3 weeks at 55ºC grown on ground filter paper. The strains of *Thermoactinomyces sacchari* 25 *thermoactinomyces fusca(34)* and *Streptomyces thermofuscus* were the most active strains in producing of reducing sugars as a result of cellulase activity reached 0 – 65 – 0.95 ,0.85-0.96 and 0.75 – 0.95 gm/ml reducing sugars measured by C_1 and C_x respectively. These results are in harmony with the findings of Mondels and Weber 1969; Bhat and Bhat (1997); who stated that thermoactinomyces are the most active cellulose-decomposing microorganism during the thermophilic phase of decomposition.

Table (6): Production of C_1 & C_x cellulose by the most active cellulolytic different strains of thermophilic actinomycetes grown on ground filter paper.

Strains	Reducing sugars mg/mL Incubation periods – week at 55ºC					
	1		2		3	
	C_1	C_x	C_1	C_x	C_1	C_x
Thermoactinomyces Sacchari (25)	0.42	0.81	0.53	0.92	0.65	0.95
Thermoactinomyces vulgaris (17)	0.21	0.43	0.42	0.53	0.56	0.76
Thermoactinomyces fusca (34)	0.47	0.77	0.65	0.92	0.85	0.96
Thermomonospora alba (27)	0.32	0.61	0.47	0.75	0.55	0.81
Streptomyces thermofuscus (30)	0.42	0.62	0.56	0.92	0.75	0.97

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تعريف بعض العزلات المنتقاة من الاكتينوميسيتات المحبة للحرارة العالية أثناء تخزين السماد البلدي

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تم تخزين كومه من السماد البلدى بأبعاد 1.5 × 2 × 2)متر (بهدف عزل الاكتينوميسنات المحبة للحرارة العالية خلال فترات التخزين حتي 100يوم فقد تم عد كل من العدد الكلي للبكتريا والبكتيريا المتجرثمة والبكتريا المحللة السليلوز الهوائية والفطريات والاكتينوميسيتات الميزوفيلية والثرموفيلية حيث وصلت درجة الحرارة علي عمق 60سم أعلي معدلات لها 70°م بعد الأربع أسابيع الأولي من التخزين .أما بالنسبة للتغير في رقم PHاستمر في النقص إلي نهاية التجربة .ودراسة قدرتها علي سرعة تحليل المخلفات الزراعية بطريقة آمنه ليس لها تأثير علي تلوث البيئة.

ويمكن تلخيص النتائج المتحصل عليها فيما يلي:

تم تقدير كل من العدد الكلي للبكتريا و البكتريا المتجرثمة و البكتريا المحللة للسليولوز الهوائية و الفطريات وكذلك الأكتينومايسيتات الميزوفيلية و الثرموفيلية .وقد سجلت البكتريا أعدادا عالية في بداية التخزين ثم أخذت في النقص إلي نهاية فترة التخزين، أما البكتريا المتجرثمة فقد سجلت أعلي أعداد بعد نهاية الأسبوع السادس وبالنسبة للبكتريا المحللة السليلوز الهوائية كانت هناك زيادة بسيطة من الأسبوع الرابع وصلت أعلاها في الأسبوع السادس ثم أخذت في النقص إلي نهاية الفترة وكذلك الفطريات فقد أخذت نفس الاتجاه في أعدادها .وسجلت أعداد الأكتينوميسيتات الميزوفيلية عموما زيادة في الأعداد عن الثرموفيلية في بداية التحلل ومع تقدم عملية التحلل ومع زيادة درجة الحرارة اتجهت أعداد الأنواع الثرموفيلية إلي الزيادة حيث وصلت أقصاها من الأسبوع الرابع حتي السادس بينما كانت الأعداد الميزوفيلية غير متواجدة إلا في الفترة الأخيرة فقط .تم عزل 60عزلة تنتمي إلي الأكتينوميسيتس واستخدم تقسيم برجي 1998لتصنيفها إلي أجناس والأنواع التابعة لها حيث كانت الأجناس تنتمي إلي كل من ثرمو أكتينوميسيس 29)عزلة (وثرمومونوسبورا 21)عزلة (والأستربتوميسيس 10)عزلات(، بالنسبة لتصنيف الأجناس إلي الأنواع فقد كانت كالتالي:

ثرمواكتينوميسيس سكاريا ، ثرمواكتينوميسيس فولجارس، ثرمومونوسبورا فيوسكا وثرمومونوسبورا ألبا، إستربتوميسيس ثرموفيوسكاس، وكانت أعلاها بالنسبة للكفاءة في تحليل السليلوز هي ثرموا اكتينوميسيس سكاريا وثرمونوسبورا فيوسكا يليها استربتوميسيس ترموفيوسكاس وتساوت كل من الثرموأكتينوميسيس فولجارس ، ثرمومونوسبورا ألبا .وكانت أكثر العزلات أنتاجاً لإنزيما السليوليزس C_1 C_Xعلي التوالي هي ثرمومونوسبورا فيوسكا، ثرمواكتينوميسيس سكاريا ، أستربتوميسس ، ثرموفيوسكاس وترمو اكتينوميسيس فولجارس ثم ثرمومونوسبورا ألبا.