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Estimation of nitrogen mineralization by nitrifying bacterial isolates *btrs8* and *nbtrs2* from *bt* and *nbt* rhizospheric soil of cotton plant by using different carbon sources

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Abstract

Numerous direct and indirect environmental factors influence the species and functional diversity of microbial communities in the soil. The physiological conditions play an important role in the growth of the organisms, present research were carried out to observe the pair wise difference of carbon sources with respect to the incubation time for both *Bacillus thuringensis* rhizospheric soil (*BtRs8*) and non*Bacillus thuringensis* rhizospheric soil (*NBtRs2*) isolates from *Bacillus thuringensis* (*Bt*) and *Bacillus thuringensis* (*NBt*) cotton rhizospheric soil for mineralization of Nitrogen to Nitrites and Nitrates. Nitrite production by *Bt* and *NBt* isolates was observed by incubating upto 9 days, whereas for nitrate production by *Bt* and *NBt* isolates were incubated up to 14 days. Sodium acetate identified as the best carbon source with a maximum production of nitrite for both of the isolates but *NBt* showed maximum production.

1. Introduction

Soil is one of the great scientific frontiers and the rhizosphere is being the most active portion of that frontier in which biogeochemical reactions influence a host of landscape and global scale processes. A better understanding of these changes is critical for maintaining the soil, thus the health of the planet for sustaining on it (Morrissey *et al*, 2004). During the nitrogen mineralization process the termination of the reactions occur at the point where ammonium is formed. This is the most reduced form of nitrogen and serves as starting point for the process known as nitrification, the biological formation of nitrite or nitrate forms contained reduced nitrogen (Arp *et al*, 2002). In certain conditions, two separate and distinct steps are distinguishable in nitrification. Since nitrate frequently appear during ammonium oxidation, it seemed apparent to early microbiologists that the transformation involves an initial oxidation to nitrite and later to nitrate. Estimation of the rate of nitrogen mineralization is the gain of information on available nitrogen to plants. The immediate product of nitrogen mineralization is ammonia (ammonium ion), generated by ammonification which in turn gets converted to nitrate by nitrification depending on the prevailing redox conditions and suitable microorganisms i.e. wide spectrum of bacteria and fungi which are naturally involved in nitrogen mineralization.

Numerous direct and indirect environmental factors influence the species and functional diversity of microbial communities in the soil. The physiological conditions play an important role in the growth of the organisms. Incubation temperature, pH, NaCl concentration, Carbon, Nitrogen sources and C/N ratio influences the metabolic reactions through enzymatic activities thereby effecting the growth of the organisms.

Methodology

Estimation of Nitrite in Acc

Nitrite (-N) was estimated by diazotization following the method of Barnes and Folkard (1951). Suitable aliquots from the culture were pipetted into test tubes and 1 ml of 1 % Sulphanilamide in 1N HCl was added and shaken thoroughly. To the so formed coloured diazo compound, 1 ml of 0.12 % N-(1-naphthyl) ethylene diaminedihydrochloride in distilled water was added. Absorbance of pink coloured solution was read at 520 nm in a UV-Vis

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Spectrophotometer - 117 (Systronics). The amount of nitrite was calculated by referring to a calibration curve prepared with the standard solution of known nitrite concentrations.

(Systronics). The amount of nitrate was calculated by referring to a calibration curve prepared with the standard solution of known nitrate concentrations.

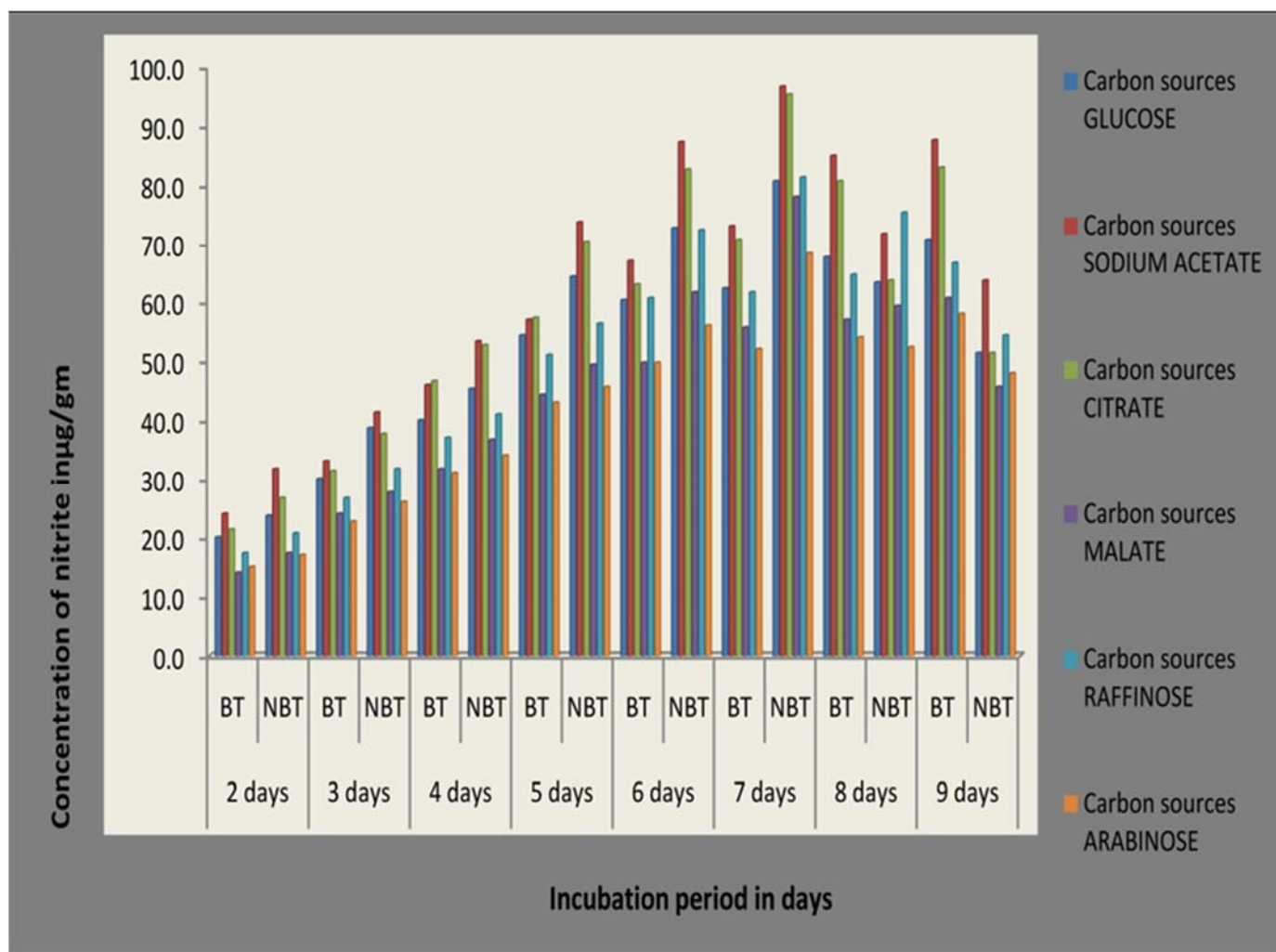
Estimation of Nitrate in Nitrite Calcium Carbonate (NCC) Medium

The nitrate (NO₃⁻-N) produced due to the activity of microorganisms in NCC medium was determined by the method of Ranney and Bartlett (1972). Three drops of brucine reagent (2 g brucine dissolved in 50 ml ethanol) were added to suitable aliquots of the culture tubes followed by 2 ml of concentrated Sulfuric acid. The solution was mixed by vortexing and placed in the dark for 30 minutes to ensure full colour development, after which the volume was made up to 10 ml with distilled water and the developed yellow colour was read at 410 nm in a UV-Vis spectrophotometer - 117

Effect of Carbon Sources on Nitrification Activity

Nitrogen mineralization was detected by estimating the initial ammonium and oxidized forms, the nitrite and nitrate. Different carbon sources (1% w/v) like Glucose, Sodium acetate, Raffinose, Malate, Arabinose and Citrate were amended in 250 ml of Basal mineral salts medium to find out suitable carbon source for the production of nitrite and nitrate. Culture flask without carbon source was the control (Eric Allen Strauss, 2000).

Results



Effect of Carbon Sources on Nitrite Production of Bt And Nbt Isolates

Table: 1 Mean and Standard Deviation of carbon sources on nitrite production of Bt and Nbt isolates

INCUBATION DAY	ORGANISMS	CARBON SOURCE						
		GLUCOSE	SODIUM ACETATE	CITRATE	MALATE	RAFFINOSE	ARABINOSE	TOTAL
2	BT	20 ± 0	24 ± 0	21.33 ± 0.58	14 ± 1	17.33 ± 0.58	15 ± 0	18.61 ± 3.65
	NBT	23.67 ± 0.58	31.67 ± 0.58	26.67 ± 0.58	17.33 ± 0.58	20.67 ± 0.58	17 ± 0	22.83 ± 5.37
3	BT	30 ± 0	33 ± 0	31.33 ± 0.58	24 ± 1	26.67 ± 1.15	22.67 ± 0.58	27.94 ± 3.95
	NBT	38.67 ± 0.58	41.33 ± 0.58	37.67 ± 0.58	27.67 ± 0.58	31.67 ± 0.58	26 ± 0	33.83 ± 5.93
4	BT	40 ± 0	46 ± 0	46.67 ± 0.58	31.67 ± 0.58	37 ± 1	31 ± 0	38.72 ± 6.39
	NBT	45.33 ± 0.58	53.33 ± 0.58	52.67 ± 0.58	36.67 ± 0.58	41 ± 0	34 ± 0	43.83 ± 7.6
5	BT	54.33 ± 1.15	57 ± 0	57.33 ± 0.58	44.33 ± 0.58	51 ± 1	43 ± 0	51.17 ± 5.9
	NBT	64.33 ± 0.58	73.67 ± 0.58	70.33 ± 0.58	49.33 ± 0.58	56.33 ± 0.58	45.67 ± 0.58	59.94 ± 10.68
6	BT	60.33 ± 0.58	67 ± 0	63 ± 1	49.67 ± 0.58	60.67 ± 0.58	49.67 ± 0.58	58.39 ± 6.75
	NBT	72.67 ± 0.58	87.33 ± 0.58	82.67 ± 0.58	61.67 ± 0.58	72.33 ± 0.58	56 ± 0	72.11 ± 11.22
7	BT	62.33 ± 0.58	73 ± 0	70.67 ± 0.58	55.67 ± 0.58	61.67 ± 0.58	52 ± 0	62.56 ± 7.69
	NBT	80.67 ± 0.58	96.67 ± 0.58	95.33 ± 0.58	78 ± 0	81.33 ± 0.58	68.33 ± 0.58	83.39 ± 10.18
8	BT	67.67 ± 0.58	85 ± 0	80.67 ± 0.58	57 ± 1	64.67 ± 0.58	54 ± 0	68.17 ± 11.72
	NBT	63.33 ± 0.58	71.67 ± 0.58	63.67 ± 0.58	59.33 ± 0.58	75.33 ± 1.15	52.33 ± 0.58	64.28 ± 7.83
9	BT	70.67 ± 0.58	87.67 ± 0.58	83 ± 0	60.67 ± 0.58	66.67 ± 0.58	58 ± 0	71.11 ± 11.25
	NBT	51.33 ± 0.58	63.67 ± 0.58	51.33 ± 0.58	45.67 ± 0.58	54.33 ± 0.58	48 ± 0	52.39 ± 5.92

Table: 2 Summary of Repeated Measures ANOVA for carbon sources on nitrite Production of Bt and Nbt

Source	Sum of Squares	df	Mean Square	F-value	p-value
days	88,707.080	7	12,672.440	38826.19**	0.000
days * Carbon sources	1,420.399	35	40.583	124.338**	0.000
days * Organisms	8,839.358	7	1,262.765	3868.89**	0.000
days * Carbon sources * Organisms	1,760.955	35	50.313	154.150**	0.000
Error(days)	54.833	168	0.326		
** significant at 1% level					

Table: 3 Tests of Between-Subjects Effects for carbon sources on nitrite

Source	Sum of Squares	df	Mean Square	F-value	p-value
Carbon sources	14,252.017	5	2,850.403	9545.537**	0.000
Organisms	1,453.503	1	1,453.503	4867.54**	0.000
Carbon sources * Organisms	101.351	5	20.270	67.88**	0.000
Error	7.167	24	0.299		

** Significant at 1% level

Table: 4 Duncan's Multiple Range test for carbon sources on nitrite Production of Bt and NBt

Duncan's Multiple Range test							
Carbon sources	N	Subset					
		1	2	3	4	5	6
Arabinose	6	42.04					
Malate	6		44.54				
Raffinose	6			51.17			
Glucose	6				52.83		
Citrate	6					58.40	
Sodium Acetate	6						62.00

Effect of Carbon Sources on Nitrite Production of Bt And Nbt Isolates

Mean and standard deviations of nitrite content have been summarized for different days according to various carbon sources between Bt and NBt separately in the (Table 1 and Fig 1). Further, Repeated measures ANOVA has been conducted by means of days, carbon sources and organisms simultaneously .From summary, it can be concluded that there is a significant impact of days on nitrite, interaction Effect of days & organisms, interaction effect of days & carbon sources and also interaction effect of days, carbon &

Organisms on nitrite content. Moreover, the results reveal that there is a significant difference among various carbons, between Bt and NBt and also among interactions of Bt, NBt with carbon over the observed period (9 days) on nitrite content. Further DMRT results suggests that various carbon sources were significantly deviating from one another with regard to nitrite content, in which sodium acetate provides the highest nitrite value 62.00µg/g, citrate provides 58.40, glucose 52.83, raffinose 51.17, malate 44.54 and arabinose provides the lowest nitrite value 42.04 µg/g.

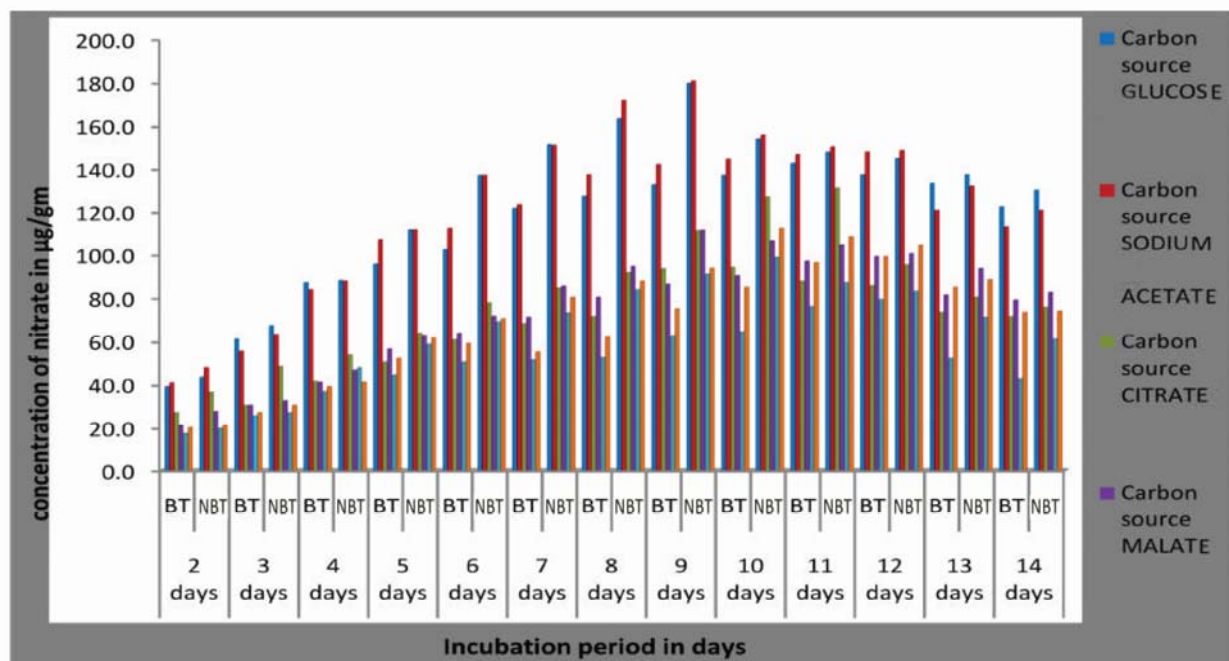


Fig 2. Effect of carbon sources on nitrate production of Bt and NBt isolates

Table: 5 Effect of carbon sources on nitrate production of Bt and NBT isolates

INCUBATION DAY	ORGANISMS	CARBON SOURCE						
		GLUCOSE	SODIUM ACETATE	CITRATE	MALATE	RAFFINOSE	ARABINOSE	TOTAL
2	BT	39 ± 1	40.67 ± 0.58	27 ± 1	21 ± 1	17.33 ± 0.58	20 ± 1	27.5 ± 9.49
	NBT	43.33 ± 0.58	47.67 ± 0.58	36.33 ± 0.58	27.5 ± 0.71	19.67 ± 0.58	21 ± 1	32.88 ± 11.26
3	BT	61 ± 1	55.33 ± 0.58	30.33 ± 0.58	30.33 ± 0.58	25.33 ± 0.58	27 ± 1	38.22 ± 14.74
	NBT	67 ± 1	63 ± 1	48.33 ± 0.58	32.5 ± 0.71	27 ± 1	30.33 ± 0.58	45.41 ± 16.57
4	BT	87 ± 1	83.67 ± 0.58	41.33 ± 0.58	41 ± 1	36.67 ± 1.15	39 ± 1	54.78 ± 22.32
	NBT	88 ± 1	87.67 ± 0.58	53.67 ± 0.58	46.5 ± 0.71	47.67 ± 0.58	41 ± 1	61.59 ± 20.37
5	BT	95.67 ± 0.58	107 ± 1	50.33 ± 0.58	56.33 ± 0.58	44.33 ± 0.58	52 ± 1	67.61 ± 25.04
	NBT	111.67 ± 0.58	111.67 ± 0.58	63.67 ± 0.58	62.5 ± 0.71	58.67 ± 0.58	61.33 ± 0.58	79.18 ± 24.79
6	BT	102.33 ± 0.58	112.33 ± 0.58	60.67 ± 0.58	63.67 ± 0.58	50.33 ± 0.58	59 ± 1	74.72 ± 24.28
	NBT	137 ± 1	137 ± 1	77.67 ± 0.58	71.5 ± 0.71	69 ± 1	70.33 ± 0.58	95.06 ± 32.07
7	BT	121.67 ± 2.08	123.33 ± 0.58	68 ± 0	71 ± 1	51.33 ± 0.58	55 ± 1	81.72 ± 30.5
	NBT	151.33 ± 0.58	151 ± 1	84.67 ± 0.58	85.5 ± 0.71	73 ± 1	80.33 ± 0.58	105.41 ± 35.08
8	BT	127.33 ± 0.58	137.33 ± 0.58	71.33 ± 0.58	80.33 ± 0.58	52.33 ± 0.58	62 ± 1	88.44 ± 33.25
	NBT	163.33 ± 0.58	171.67 ± 0.58	91.67 ± 0.58	94.5 ± 0.71	83.67 ± 0.58	87.67 ± 0.58	116.65 ± 38.94
9	BT	132.67 ± 1.15	142 ± 0	93.33 ± 0.58	86.33 ± 0.58	62.33 ± 0.58	75 ± 1	98.61 ± 29.99
	NBT	179.67 ± 0.58	180.67 ± 0.58	111.33 ± 0.58	111.5 ± 0.71	91 ± 1	93.67 ± 0.58	128.94 ± 39.8
10	BT	137 ± 1	144.67 ± 0.58	94 ± 5.29	90.33 ± 0.58	64.33 ± 0.58	85 ± 1	102.56 ± 29.62
	NBT	154 ± 0	155.67 ± 0.58	127 ± 1	106.5 ± 0.71	98.67 ± 0.58	112.33 ± 0.58	126.82 ± 23.12
11	BT	142.67 ± 0.58	146.67 ± 0.58	87.67 ± 1.15	97 ± 1	76 ± 0	96.33 ± 0.58	107.72 ± 27.84
	NBT	148 ± 1	150.33 ± 0.58	131 ± 1	104.5 ± 0.71	87 ± 1	108.33 ± 0.58	122.53 ± 24.41
12	BT	137.33 ± 0.58	148 ± 0	85.67 ± 0.58	99 ± 1	79.33 ± 0.58	99 ± 1	108.06 ± 26.39
	NBT	145 ± 1	148.67 ± 0.58	95.33 ± 0.58	100.5 ± 0.71	83 ± 1	104.33 ± 0.58	113.53 ± 26.3
13	BT	133.33 ± 1.15	120.67 ± 0.58	73.33 ± 0.58	81.33 ± 0.58	52 ± 0	85 ± 1	90.94 ± 28.61
	NBT	137.33 ± 0.58	132 ± 0	80.33 ± 0.58	93.5 ± 0.71	71 ± 1	88.33 ± 0.58	100.82 ± 26.72
14	BT	122.33 ± 1.53	113 ± 0	71.33 ± 0.58	79 ± 1	42.67 ± 0.58	73.33 ± 0.58	83.61 ± 27.61
	NBT	130 ± 1	120.67 ± 0.58	75.67 ± 0.58	82.5 ± 0.71	61 ± 1	74 ± 1	91.12 ± 26.96

Table: 6 Summary of Repeated Measures ANOVA for carbon sources on nitrate production on Bt and NBt

Source	Sum of Squares	df	Mean Square	F-value	p-value
days	326,122.142	12	27,176.845	50769.101**	0.000
days * Carbon sources	27,967.634	60	466.127	870.772**	0.000
days * Organisms	8,233.354	12	686.113	1281.72**	0.000
days * Carbon sources * Organisms	5,095.912	60	84.932	158.661**	0.000
Error(days)	147.744	276	0.535		

** Significant at 1% level

Table: 7 Tests of Between-Subjects Effects for carbon sources on nitrate production on Bt and NBt

Source	Sum of Squares	df	Mean Square	F-value	p-value
Carbon sources	278,223.510	5	55,644.702	15219.78**	0.000
Organisms	22,576.013	1	22,576.013	6174.93**	0.000
Carbon sources * Organisms	922.382	5	184.476	50.457**	0.000
Error	84.090	23	3.656		

** Significant at 1% level

Table: 8 Duncan's Multiple Range test for carbon sources on nitrate Production on Bt and NBt

Duncan's Multiple Range test							
Duncan							
Carbon sources	N	Subset					
		1	2	3	4	5	6
Raffinose	6	58.64					
Arabinose	6		69.26				
Malate	5			72.75			
Citrate	6				74.27		
Glucose	6					119.04	
Sodium Acetate	6						120.47

Mean and standard deviations of nitrate content have been summarized for different days according to various carbon sources between Bt and NBt separately in the (Table5). Further Repeated measures ANOVA has been conducted by means of days, carbon sources and organisms simultaneously (Table 6). From the first (Table 6) of summary, it can be concluded that there is a significant impact of days on nitrate content, interaction effect of days & organisms, interaction effect of days & carbon sources and also interaction effect of days, carbon & organisms on nitrate. Moreover, the results (Table 7) reveal that there is a significant difference among various carbon, between Bt and NBt and also among interactions of Bt, NBt with carbon over the observed period (14 days) on nitrate content. Further DMRT (Table 8) suggests that various carbon sources are significantly deviating from one another with regard to nitrate content, in which sodium acetate provides the highest nitrate value 120.47 µg/g, glucose 119.04, citrate 74.27, malate 72.25, arabinose 69.26 and raffinose provide the lowest nitrate value 58.64 µg/g.

Discussion

In the present study after selection of suitable media, for production of nitrites and nitrates the isolates were tested by using standard protocols (Barnes and Folkard, 1951; Jackson, 1971; Ranney and Bartlett, 1972). The change in concentration of exchangeable forms of NH_4^+ , N_2 , NO_2^- , N^- and NO_3^- during the nitrification process was studied up to 14 days. It was observed that nitrite production in ACC medium by *Bacillus cereus* increased with incubation time

upto 9th day and later a gradual decrease was observed. The nitrate production in NCC medium was observed to increase upto 12th day and later there was a gradual decrease. Similar results were reported by (Ramakrishna and Sethunathan, 1983).

If the carbon skeleton is not recycled, there should be a relationship between the amount of carbon utilized and the nitrite/nitrate released. The amount of nitrite accumulated was proportional to the initial sodium acetate concentration. However, up to now there is no convincing explanation why nitrite is produced only with acetate, citrate, malate, and maltose while several other C-sources supported growth but not nitrification. Similar carbon dependencies of heterotrophic nitrification have been reported (Hyllin and Matsumoto, 1960; Verstraete and Alexander, 1972b), but the carbon sources favoring nitrification are not unique among different organisms. This may be explained by the regulative properties of the key enzymes in the glyoxylate cycle (Kornberg, 1966). These enzymes may be induced by acetate and repressed by glucose, PEP or pyruvate, (Donawa and Inness, 1970; Kleber and Mtiller, 1970; Hanozet and Guerritore, 1972; Wolfson and Grulwich, 1972).

Further, Repeated measures ANOVA and Duncan's Multiple Range Test (DMRT) were carried out to observe the pair wise difference of carbon sources with respect to the incubation time for both Bt and NBt isolates for mineralization of Nitrogen to Nitrites and Nitrates. Nitrite production by Bt and NBt isolates was observed by incubating upto 9 days, whereas for nitrate production by Bt and NBt isolates were incubated up to 14 days. Sodium

acetate identified as the best carbon source with a maximum production of nitrite for both of the isolates but NBt showed maximum production 96.7 µg by day 7, and Bt shows maximum production 87.67 µg by day 9 and arabinose provided the lowest nitrite value. Sodium acetate provided highest nitrate value for both isolates NBt shows maximum production 180.67 µg by day 9 and Bt shows maximum production 148 µg by day 12 and raffinose provided the lowest nitrate value, and were found significant. The result suggested that the difference between any two carbon sources with respect to the incubation time was significant which means the nitrate production levels were varying according to the carbon source incubation in a medium containing sodium acetate.

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