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GROWTH OF *BACILLUS THURINGIENSIS* ISOLATES (B.t.-16 and B.t.-64) ON ORGANIC SUBSTRATES AS NUTRIENT SOURCE

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ABSTRACT

Different organic substrates (maize flour, barley flour, rice flour, sawdust, wheat bran and millet grains) were used as nutrient source to *Bacillus thuringiensis* Berliner (B.t.) isolates (B.t.-16 and B.t.-64). The purpose of the study was to determine the most efficient organic substrate least expensive for the growth of the bacteria. All the tested substrates showed increase in growth of B.t. isolates from the first week except sawdust, which showed a decline in bacterial population in the first week but thereafter a gradual increase in bacterial population occurred till the end of the experiment. Rice, Wheat bran, and Barley flours were found to be more effective substrates for the bacterial growth. The isolate B.t.-16 appeared to possess relatively more ligninolytic potential. Based on promotion or reduction magnitude in final population sizes (on 28- day of incubation) the growth pattern of B.t.-16 and B.t.-64 on various substrates, in decreasing order of suitability, may be shown as follows:

B.t.- 16 - Rice > Wheat > Barley > Maize > Millet > Sawdust

B.t.- 64 –Wheat > Barley > Millet > Rice > Maize > Sawdust

Key words: *Bacillus thuringiensis* Berliner, isolates-B.t.-16 and B.t.-64, Bacterial growth, Nutrient substrates, ligninolytic potential

INTRIODUCTION

Bacillus thuringiensis Berliner, a Gram-positive spore-forming bacterium, is the most widely used biological agent in controlling insect pests (Sharma, 1994; Bernhard *et al.*, 1997; Nester *et al.,* 2002; Wei *et al*., 2003). Organic sources including proteins, nucleic acid and co-enzyme constituents are used for optimal growth and production of *B. thuringiensis.* Sakharova *et al.* (1988) studied elevated concentration of glucose, yeast extract and acetate on growth of *B. thuringiensis* subsp. *galleriae*. The ingredients of these media are expensive and not feasible for use at large scale production. There is, therefore, a need to use less expensive media for growth and production of *B. thuringiensis*. Less expensive agricultural products such as cereal, legume and oil seeds have been used as source of nutrient for the growth of *B. thuringiensis*. (Abdel-Hameed *et al.*, 1990). Powder of green gram and other leguminous seeds, was used as protein source along with combination of soluble starch and or cane sugar molasses as carbohydrate source for the production of delta endotoxins (Mummigatti and Raghunathan, 1990). Defatted ground nut cakes as first nitrogen source and gram flour, soybean and defatted milk powder as the second source for production of *B. thuringiensis var. islariensis* have also been tested by Desai and Shethna (1991). Coconut water, fishmeal and rice bran have been used for the production of *B. thuringiensis* by Lee and Seleena (1991). Less expensive sources of organic nitrogen such as legumes, cereals, animal proteins, leaf proteins, oilseeds, yeast and tubers have been studied for the production of *B thuringiensis* subsp. *aizawai* (Morris *et al*., 1997). Tirado-Montiel *et al.* (2001) used wastewater treatment sludge for the growth of *B. thuringiensis*. Lauria Bertani + salt + 0.2% glucose, 1.5% glucose + 0.5% soybean flour + salts, Liquid swine manure 4% + 0.2% glucose have been experimented as nutrient source for production of *Bacillus thuringiensis* by Valicente *et al*. (2010). Soybean flour, Groundnut cake powder, Wheat bran and Nutrient yeast salt medium (NYSM) were also used as less expensive sources of nutrients for the production of *Bacillus thuringiensis* var *Israelensis* (Prabakaran and Balaraman, 2006). In the present studies, Maize, Rice, Barley flours, Wheat bran, Millet grains and Sawdust have been evaluated for the growth of *Bacillus thuringiensis isolates*, B.t. -16 and B.t.- 64.

MATERIALS AND METHODS

Growth of *B. thuringiensis* isolates BT-16 and BT-64 was studied on different organic substrates (Maize, Rice, and Barley flours, Wheat bran, Millet grains and sawdust). Twenty five g of organic substrate was taken in 250 mL conical flasks. Moisture level of 60% Maximum water holding capacity of the substrate (MWHC) was maintained by adding sterilized water. Flasks were plugged with cotton. The flasks were then autoclaved at 121ºC at 15 psi for 15 minutes. After sterilization 1mL of BT-16 or BT-64 suspension containing Log cfu 8.2778 and Log cfu 8.6396, respectively was added in the flask. Each treatment was replicated three times. Observations were taken after 0, 7, 14, 21 and 28 days by suspending 1g substrate in 9mL sterile distilled water. Serial dilutions were made and 1mL bacterial suspension from the appropriate dilution was poured into Petri plates and Lauria Bertani agar (autoclaved and cooled) was poured in each Petri plate. The plates were than incubated at 30 ± 2°C. Bacterial colonies that were formed after 24 h were counted as colony forming units ($CFU.mL^{-1}$) and expressed as $logCFU.g^{-1}$ substrate.

The nutrient characteristics of the substrates are outlined in Table 1. Barley flour, Wheat bran and Millet grains have protein in larger proportion than maize and rice flours whereas rice and maize have relatively higher concentrations of carbohydrates. Wheat bran is comparatively much richer in K, P, Mg and Se and vitamins than other substrates. Whole millet grains follow wheat bran as regards to their nutritive quality. Saw dust contains cellulose, lignin and ash and also non hydrolyzed polysaccharides, sugar and some organic acids.

Table 1. Nutrient characteristics of the substrates (per 100g). *

Nutrient Components	Maize Flour	Barley Flour	Rice Flour	Wheat Bran crude	Millet grains	Sawdust = Wood	
Water (%)	10.91	12.11	11.89	9.89	8.67	Cellulose	HW – 40-44
Energy (kJ)	1510	1443	1531	904	1582		SW – 40-44
Proteins (g)	6.93	10.50	5.95	15.5	11.02	Hemi-cellulose	HW- 15-35
Lipids (FAT) (g)	3.86	1.60	1.42	4.25	4.22		SW– 20.32
Carbohydrate (By diff.) (g)	76.85	74.52	80.13	64.51	72.85	Lignin	HW -18-25 SW – 25-35
Ash (g)	1.45	1.28	0.61	5.79	3.25		
Fiber (g)	7.2	10.1	2.4	42.8	8.5	HW : Hardwood SW : Softwood	
Sugar (g)	7.64	0.8	0.12	0.41	-		
Ca (mg)	7	32	10	73	8		
Mg (mg)	93	2.68	0.35	611	3.01		
P (mg)	2.72	96	35	1013	114		
Fe (mg)	2.38	296	98	10.57	285		
K (mg)	315	309	76	1182	195		
Na (mg)	5	4	0	2	5		
Zn (mg)	1.73	1.034	0.8	7.27	1.68		
Cu (mg)	0.23	2	0.130	0.998	0.75		
Mn (mg)	0.46	0.343	1.20	11.50	1632		
Se (mcg)	15.4	37.7	15.10	77.6	2.7		
Thiamin (mg)	0.246	0.37	0.138	0.523	0.421		
Riboflavin (mg)	0.08	0.114	0.021	0.577	0.290		
Niacin (mg)	1.9	6.269	2.59	13.578	4.72		
Pantothenic acid (mg)	0.658	0.145	0.819	2.181	0.848		
Vitamin B_6 (mg)	0.37	0.396	0.436	1.305	0.384		
A-Tocopherol (mg)	0.42	0.57	0.11	1.49	0.05		

*, USDA (2010). National Nutrient Data Base for Standard Reference. Release 23.

Table 2. Two-way ANOVA for complete randomized design of interaction of substrates and incubation period with the growth of Bt.16 (expressed as $Log_{10}\ CFU.g^{-1}$ substrate).

Source	SS	Df	MS	F	P
Time (T)	30.26313	4	7.56578	418.577	0.0001
Substrates	16.00827	5	3.20165	177.13171	0.0001
T x Subs	10.24956	20	0.51247	28.35287	0.0001
Error	1.08449	60	0.01807	-	-
Total	57.60546	89	-	-	-
Time $LSD_{0.05}$: 0.08964; Substrate $LSD_{0.05}$: 0.098198					

Table 3. Two-way ANOVA for complete randomized design of interaction of substrates and incubation period with the growth of Bt.64. (expressed as Log $_{10}$ CFU.g^{-1} substrate).

Source	SS	Df	MS	F	p
Time (T)	10.99644	4	2.74911	598.99625	0.0001
Substrates	26.38970	5	5.27794	1149.99586	0.0001
T x Subs	13.27201	20	0.66360	144.59013	0.0001
Error	0.27537	60	0.004589	-	
Total	50.93353	89			
Time LSD $_{0.05}$: 0.04517; Substrate LSD $_{0.05}$: 0.0.049482					

RESULTS AND DISCUSSION

The populations of *B. thuringiensis* isolates- B.t.-16 and B.t.-64, expressed as $\log_{10}$.CFU.g^{-1} on various substrates were weekly monitored for four weeks of incubation at 30 ^{o}C. Both of the factors, the substrate types and the period of incubation, influenced the growth of both isolates significantly ($p < 0.0001$) as indicated by the two – way ANOVA of the growth data (Table 2 and 3). The two factors also interacted significantly ($p < 0.0001$) with each other. The population dynamics of the two isolates on various substrates may be described as follows:

***B. Thuringiensis* isolate - B.t.-16**

When growing on maize flour, B.t.-16 exhibited two peaks of growth – one at the 7th day from log units 8.2778 to 9.5778 and other peak at 28th day of incubation (Fig. 1). After one week of incubation, its population increased by 1.3 log units, declining in next week by a quantum of 0.55 log units to 9.0203. Henceforth, the population increased regularly attaining next peak of growth on 28th day amounting to 9.592 log units. The population related with period of incubation through a third degree polynomial curve as given by the following equation:

$$\text{Log}_{10}\ \text{CFU} = 8.321397 + 0.29268\ (\text{Days}) - 0.0233579\ (\text{Days})^2 + 0.000521\ (\text{Days})^3 \pm 0.2297$$

t = 63.19 t =6.12 t = -5.38 t =5.11

p < 0.0001 p < 0.0001 p < 0.0001 p < 0.0001

N=15, $R^2 = 0.841$, Adj. $R^2 = 0.797$, F = 19.37 ($p < 0.0001$)……………………….**MAIZE FLOUR**

On barley flour, the maximum growth was observed on 21st day of incubation when it reached to log CFU 10.255 only slightly higher than that on 14th day of incubation (CFU: 10.245 log units). The population declined on 28th day by 0.259 log units as compared to the maxima of 21st day but higher than initial population by a quantum of 1.718 log units. The growth related to incubation period curvilinearly as second degree polynomial.

$$\text{Log}_{10}\ \text{CFU} = 8.175368 + 0.198714\ (\text{Days}) - 0.004732\ (\text{Days})^2 \pm 0.2304$$

t = 65.32 t =9.38 t = -6.52

p < 0.0001 p < 0.0001 p < 0.0001

N=15, $R^2 = 0.932$, Adj. $R^2 = 0.921$, F = 82.003 ($p < 0.0001$)…………………….….**BARLEY FLOUR**

B.t.-16 population increased regularly for two weeks but declined on third week. Maximum CFU size was exhibited on fourth week when it reached to 10.424 log units with a net increase in size of 2.1462 log units of CFU over initial CFU size of 8.2778 log units. The overall growth was represented by the following equation.

$$\text{Log}_{10}\ \text{CFU} = 8.247755 + 0.263629\ (\text{Days}) - 0.013774\ (\text{Days})^2 + 0.0002534\ (\text{Days})^3 \pm 0.2696$$

t = 53.71 t =4.69 t = -2.70 t =2.18

p < 0.0001 p < 0.001 p < 0.021 p < 0.058

N=15, $R^2 = 0.913$, Adj. $R^2 = 0.889$, F = 38.25 ($p < 0.0001$)…………………………**RICE FLOUR**

On sawdust, the population of B.t.-16 declined initially significantly by a quantum of 0.88 log units from log units 8.2778 to 7.401. It increased in later weeks attaining maxima on 21st day (CFU 8.969 log units) showing net increase of 0.69 log units over initial population. The population related to incubation period through a three degree polynomial model.

Log_{10} CFU = 8.222369 -0.2413566 (Days) + 0.0267738 $(\text{Days})^2$ + 0.0006223 $(\text{Days})^3 \pm 0.2861$
t = 50.13 t =-4.05 t = 4.95 t =-4.90
$p < 0.0001$ $p < 0.0001$ $p < 0.0001$ $p < 0.0001$
N=15, $R^2 = 0.824$, Adj. $R^2 = 0.776$, F = 17.14 ($p < 0.0001$)…………………….…..**SAWDUST**

On wheat bran, B.t.-16 exhibited maximum population size on 14th day (10.322 log units) which was insignificantly different from that at 21st day (10.311 log units). On 14th day of incubation, the population increase was around 2.04 log units over the initial CFU size. The curvilinear relationship of growth with incubation period was given by the following equation.

Log_{10} CFU = 8.20991 + 0.212373 (Days) – 0.0052749 $(\text{Days})^2 \pm 0.2110$
t = 71.0 t =10.95 t = -7.94
$p < 0.0001$ $p < 0.0001$ $p < 0.0001$
N=15, $R^2 = 0.944$, Adj. $R^2 = 0.934$, F = 100.544 ($p < 0.0001$)………………………**WHEAT BRAN**

On millet grains, the bacterium increased in CFU regularly up till 21st of incubation with a net increase of 1.8912 log units over initial CFU size. Population, however, declined abruptly on 28th day – a decrease by 2.233 log units over the previous week. The growth of the bacterium related to the incubation period as follows.

Log_{10} CFU = 8.2000301 + 0.199133 (Days) – 0.0059635 $(\text{Days})^2 \pm 0.3190$
t = 47.27 t = 6.78 t = -5.94
$p < 0.0001$ $p < 0.0001$ $p < 0.0001$
N=15, $R^2 = 0.807$, Adj. $R^2 = 0.774$, F = 25.02 ($p < 0.0001$)……………………… **MILLET GRAINS**

Interestingly, the final CFU size in case of each substrate remained larger than the initial CFU size. The growth was profound on rice and good on barley and wheat.

***B. thuringiensis* isolate-B.t. - 64**

The peak growth of B.t.-64 on maize flour was observed on first week of incubation when an increase of 1.275 log units over initial CFU size was evident. In later weeks the population declined significantly up till 21st day of incubation. Some increase in growth was, however, observed on 28th day of incubation when an enhancement of 0.2403 log units in CFU size took place over 21st day growth. The significant predictive equation was as follows.

Log_{10} CFU = 8.67842 + 0.318277 (Days) – 0.027662 $(\text{Days})^2$ + 0.0006064 $(\text{Days})^3 \pm 0.0.1828$
t = 82.81 t = 8.36 t = -8.007 t =7.48
$p < 0.0001$ $p < 0.0001$ $p < 0.0001$ $p < 0.0001$
N=15, $R^2 = 0.865$, Adj. $R^2 = 0.828$, F = 23.43 ($p < 0.0001$)………………………..**MAIZE FLOUR**

On barley flour, population increased almost regularly during initial two weeks. By 14th day, an increase of 1.2214 log units over initial CFU of 8.6396 was recorded. There was a decline in CFU size on 21st day of incubation but some increase was again exhibited at 28th day of incubation. The CFU size varied curvilinearly with incubation period as given below.

Log_{10} CFU = 8.687359 + 0.1133355 (Days) – 0.0027481 $(\text{Days})^2 \pm 0.1542$
t = 103.67 t = 7.99 t = -5.65
$p < 0.0001$ $p < 0.0001$ $p < 0.0001$
N=15, $R^2 = 0.905$, Adj. $R^2 = 0.889$, F = 56.92 ($p < 0.0001$)………………………..**BARLEY FLOUR**

The growth trend of B.t.-64 on rice flour was essentially similar to that on barley. Of course, the population size remained somewhat low on rice flour. CFU size on rice flour related with incubation length as given by the following equation.

Log_{10} CFU = 8.582711 + 0.2580678 (Days) – 0.0181563 $(\text{Days})^2$ + 0.003612 $(\text{Days})^3 \pm 0.2532$
t = 59.14 t =4.89 t = -3.80 t =3.22
$p < 0.0001$ $p < 0.0001$ $p < 0.0001$ $p < 0.0001$
N=15, $R^2 = 0.765$, Adj. $R^2 = 0.700$, F = 11.91 ($p < 0.0001$)………………………..**RICE FLOUR**

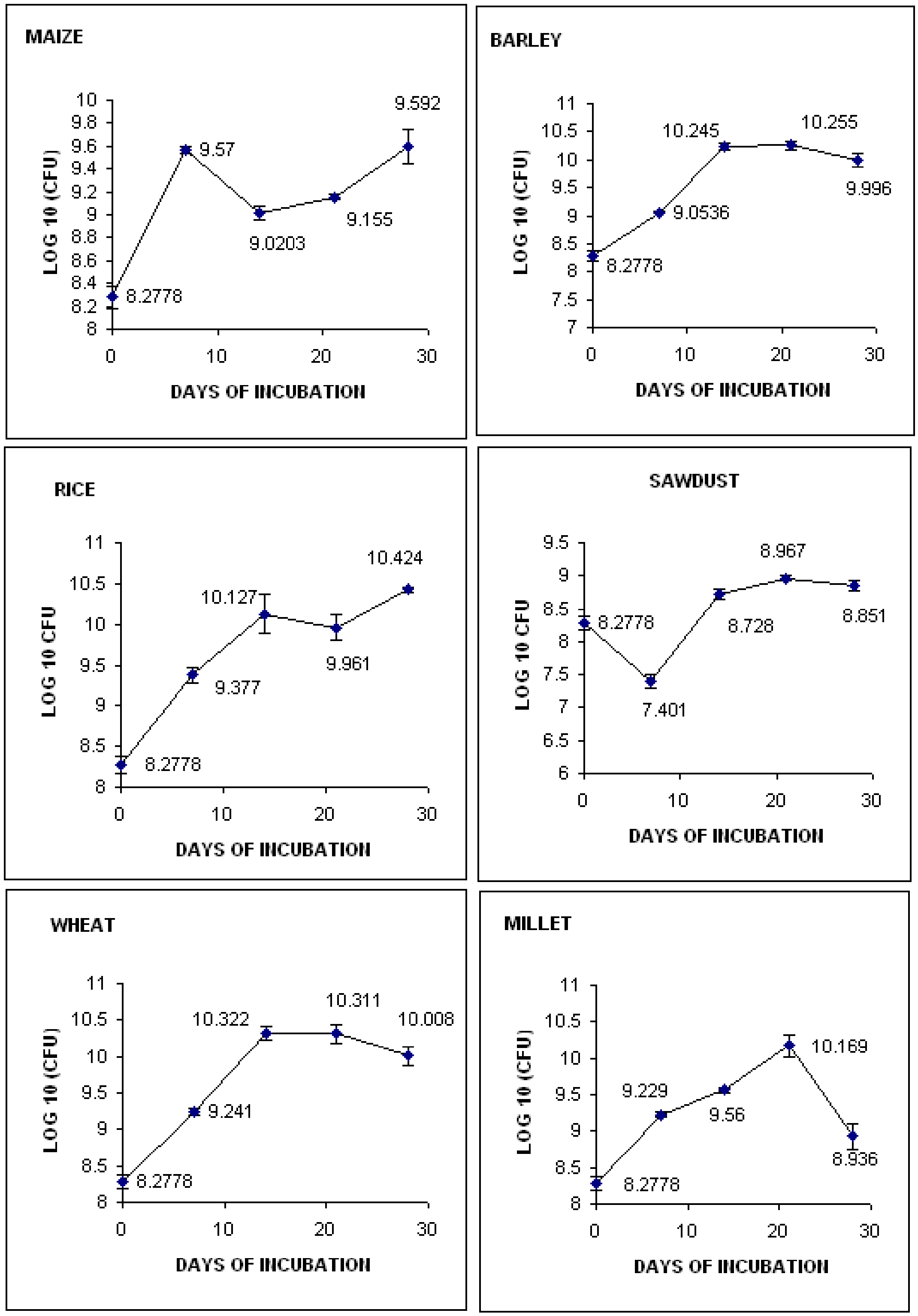

Fig. 1. Population growth of B. thuringiensis isolate B.t.- 16 (log $_{10}$ CFU .g^{-1}) up till 28 days of incubation over various nutrient substrates.

There was severe population decline of B.t.-64 of 2.6396 log units on sawdust after initial week of incubation over the initial population. The growth recovery on second and subsequent weeks was although quite substantial but the final CFU size of 8.295 log units remained lower than the initial population. The significant equation relating CFU size with the length of incubation is given below.

Log_{10} CFU = 8.531901 – 0.5532089 Days) + 0.041757 (Days)^2 - 0.0008014 (Days)^3 ± 0.4749
t = 31.34 t =-5.59 t = 4.65 t =-3.80
$p < 0.0001$ $p < 0.0001$ $p < 0.001$ $p < 0.003$
N=15, R^2 = 0.802, Adj. R^2 = 0.748, F = 14.87 ($p < 0.0001$)……………………….**SAWDUST**

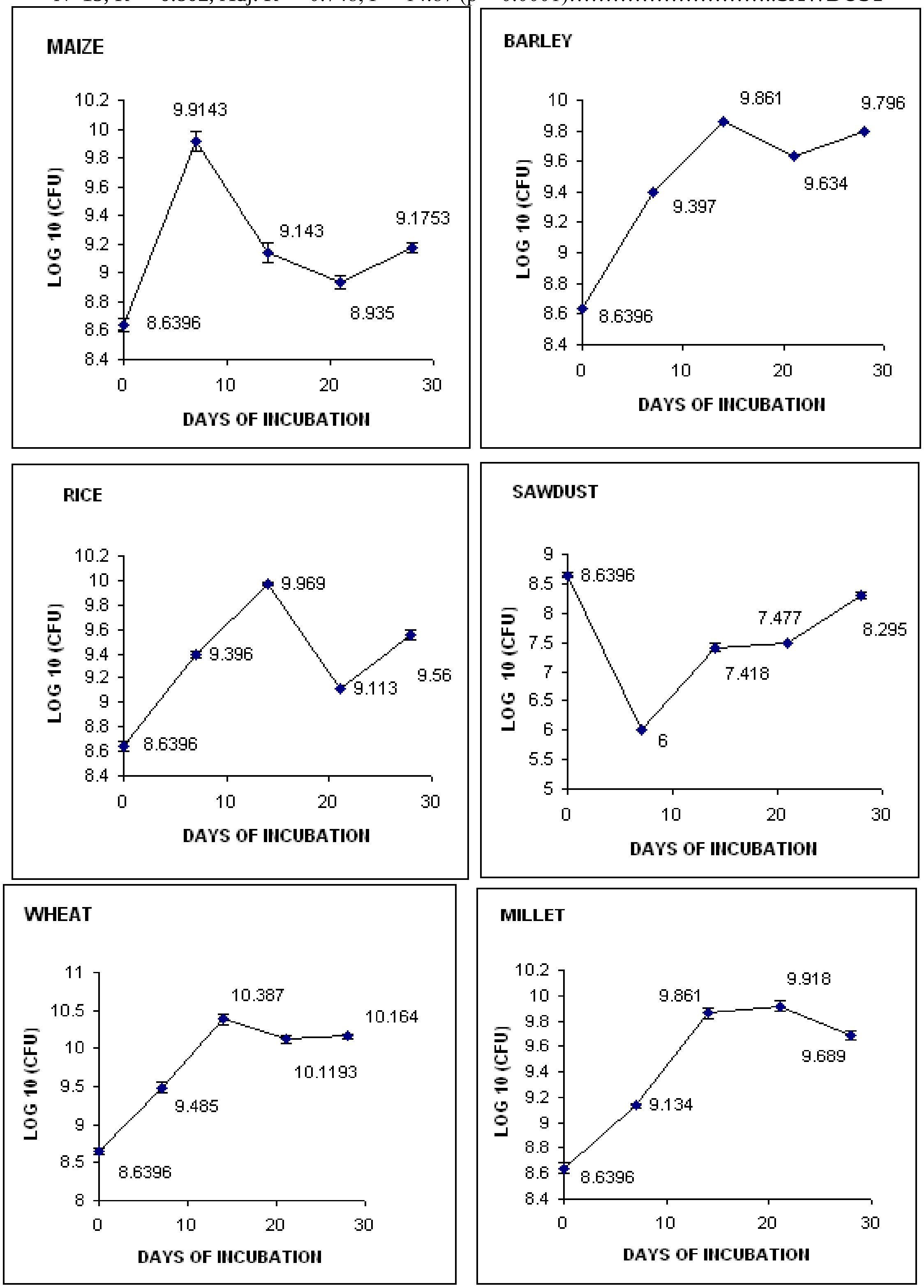

Fig. 2. Population growth of *B. thuringiensis* isolate B.t.-64 up till 28 days of incubation over different substrates.

The pattern of growth of B.t.-64 over wheat bran and millet grains was quite similar but wheat bran supported bacterial population better than the millet grains did. The growth on wheat bran was relatively faster than on any substrate. On 14th day of incubation the CFU size of 10.387 log units was the highest with net enhancement of 1.7474 log units over initial CFU size. On 21st and 28th day population declined by 0.2677 and 0.223 log units, respectively, over the maxima of the second week. On millet grains, the CFU size reached to CFU 9.861 log units on 14th day of incubation. The CFU size had increased by an insignificant quantum of 0.057 log units on 21st day. On 28th day, population decreased substantially by 0.229 log units over previous week. Following are the equations for CFU sizes on wheat bran and millet grains.

Log_{10} CFU = 8.626391 + 0.165701 (Days) – 0.0040382 (Days)^2 ± 0.1875
t = 84.67 t = 9.61 t = -6.84
p < 0.0001 p < 0.0001 p < 0.0001
N=15, R^2 = 0.931, Adj. R^2 = 0.920, F = 81.28 (p < 0.0001)……………………..**WHEAT BRAN**

Log_{10} CFU = 8.56933 + 0.1275961 (Days) – 0.003086 (Days)^2 ± 0.1272
t = 123.94 t =10.91 t = -7.71
p < 0.0001 p < 0.0001 p < 0.0001
N=15, R^2 = 0.947, Adj. R^2 = 0.938, F = 106.68 (p < 0.0001)……………………**MILLET GRAINS**

All the tested substrates, showed increase in growth of BT isolates from the first week except sawdust, which showed a decline in bacterial population in the first week but thereafter a gradual increase in bacterial population occurred till the end of the experiment. Wheat bran, Rice and Barley flours were found to be more effective substrates for the bacterial growth. Wheat bran, rice and barley have already been reported for the mass multiplication of a number of microorganisms (Rai and Rana, 1979; Rosane *et al*., 2008 and Shojaaddini *et al*., 2010). Wheat bran and rice are good source of C and N. Rice contains abundant starch and is reported to be a good nutritive media for the growth of bacteria (Das *et al*., 2008). Wheat bran contains more protein and higher amounts of K, P, Mg, Se and vitamins. The decline in B.t. population in later days of the experiment may be thought to arise as result of depletion of nutrient and competition among bacterial cells themselves owing to aggregation or due to release of some auto-inhibitory metabolic products into the medium by the bacteria.

Percent promotion or reduction in bacterial population sizes over various incubation periods and substrates for the two isolates, calculated on original (untransformed) numbers of CFU counts.g^{-1} substrate, is given in Table 4 and 5. Based on promotion or reduction of the final population sizes (on 28- day of incubation) the growth pattern of B.t.-16 and B.t.-64 on various substrates, in decreasing order of suitability, may be shown as follows:

B.t.- 16 - Rice > Wheat > Barley > Maize > Millet > Sawdust
B.t.- 64 –Wheat > Barley > Millet > Rice > Maize > Sawdust

Out of the tested substrates, the rice flour is the best substrate followed by wheat bran and barley flour for B.t.-16 and wheat bran is the best substrate followed by barley flour and millet grains for B.t.- 64. For isolate B.t.- 16, rice flour is 49.18 times better than sawdust and wheat is 20.3 times better than sawdust (Table 6). In case of B.t.-64, wheat bran is 13 times better than maize and barley flour is 5.39 times better than maize. On sawdust growth declines initially but later on bacterial growth increases. B.t.-16 appears to possess more ligninolytic activity than B.t.-64.

The decline in population after first week of incubation in both B.t.-isolates on sawdust, and thereafter a gradual promotion of their growth is interesting and may be attributed to lignin present in the sawdust and the ligninolytic capability of the bacteria. Lignin constitutes around 20-30% of dry weight of woody tissues. Lignified plant materials are known to decompose more slowly than unlignified materials. The extent of decomposition of cellulose of woody material by vigorous bacteria is reduced with the increase of lignin concentration in the substrate (Fuller and Norman, 1943). Moreover, partial decay of lignin provides numerous monomers such as ferulic and vanillic acids, etc. (plant growth inhibitors). Such compounds are traditionally obtained from petroleum. However, obtaining them from bacterial decay of lignin is an interesting field of research now days. Several strains of ligninolytic bacteria have been identified to degrade and assimilate lignin (Odier *et al.*, 1981; Pometto and Crawford, 1986; *Ball et al.*, 1989; Nishimura et al., 2006; Chandra *et al.*, 2007; El-Hanafy *et al.*, 2008). Recently, a *Bacillus* sp. (EU978470) has been identified from Egyptian soil of Kafr El Dawar (Abd-Elsalam and El-Hanafy (2009) to be ligninolytic. The growth of *B. thuringiensis* isolate B.t.- 16 on sawdust suggests for further investigations with this isolate as a potential ligninolytic agent.

Table 4. Percent Promotion or reductions in populations of *B. thuringiensis* isolate B.t.-16 over initial population (untransformed data) when cultured over different nutritive substrates for 28 days.

Days	Maize	Barley	Rice	Sawdust	Wheat	Millet
0	-	-	-	-	-	-
7	1766.1	466.26	1149.68	-86.67	782.86	749.58
14	433.15	8856.67	9232.18	176.34	10892.13	1733.03
21	616.10	9232.29	5114.01	366.64	10956.53	8006.26
28	2064.46	5363.32	13230.4	269.04	5461.37	399.92

Table 5. Percent Promotion or reductions in populations of *B. thuringiensis* isolate B.t.-64 over initial population (untransformed data) when cultured over different nutritive substrates for 28 days.

Days	Maize	Barley	Rice	Sawdust	Wheat	Millet
0	-	-	-	-	-	-
7	1769.8	475.84	467.76	-99.77	611.63	21.77
14	217.7	1566.38	2020.6	-93.39	5579.53	1566.38
21	96.95	883.99	194.83	-93.18	2927.29	1799.43
28	248.42	1338.41	733.25	-54.56	3230.86	1019.90

Table 6. Efficacy order of various substrates in relation to the substrate of lowest promotion.

EFFICACY ORDER OF SUBSTRATES IN RELATION TO SAWDUST						
B.t. - 16	Rice flour	Wheat bran	Barley flour	Maize flour	Millet grains	Sawdust
	49.18	20.30	19.94	7.67	1.49	1.0
EFFICACY ORDER OF SUBSTRATES IN RELATION TO MAIZE FLOUR						
B.t. - 64	Wheat bran	Barley flour	Millet grains	Rice flour	Maize flour	Sawdust
	13.01	5.39	4.11	2.95	1.0	Excluded due to reduction in growth

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