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Utilization of Lignocellulosic Waste for the Preparation of Nitrogenous Biofertilizer

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Abstract: This work is a part of solid waste management project. Bagasse, a lignocellulosic waste of sugarcane industry was utilized for producing the nitrogenous biofertilizer. Nitrogen fixing free living bacteria were isolated from soil samples using dilution plate method. Selection of bacteria *Azotobacter chroococcum* was made due to its capability to survive and fix the maximum nitrogen as compared to other bacteria tested in a medium in which bagasse was the only carbon source. *A. chroococcum*, *A. indicus* and *Azospirilum brasilense* were tested for nitrogen fixation from 7 to 28 days. Maximum nitrogen fixed by these bacteria was 67.81, 28.00 and 43.20 mg/L respectively. Experimental results justified that bagasse biomass with *A. chroococcum* is a good source of nitrogen and organic matter, which can be utilized as a biofertilizer.

Key words: Nitrogenous biofertilizer, lignocellulosic waste, free-living N-fixing soil bacteria, *Azotobacter* spp.

Introduction

After water, nitrogen is the second limiting factor in some fields for plant growth. According to Jensen (1965), non-symbiotic or free-living bacterial nitrogen fixing activity depends upon available organic matter as carbon source. In case of Rhizobia or symbiotic nitrogen fixation, carbon source is directly taken from roots of the host plants. But in case of free-living diazotrophs it must be present in the soil in any form of organic matter (Shawkey *et al.*, 1988; Pigakera, 1989; Maheswari and Purushothaman, 1990). Soils which are poor in organic matter do not promote growth of nitrogen fixing bacteria. Studies revealed that added carbohydrates enhance the growth of free-living diazotrophs and consequently the nitrogen fixation (Lethbridge and Davidson, 1983). *Azotobacter chroococcum* is widely distributed in cultivated soils. This organism brings about nitrogen fixation with a definite organic matter and soil combination. Carbohydrates especially sugar play an important role in nitrogen fixation. Nuntagij *et al.* (1989) studied that pH also affects the nitrogen fixation and pH close to neutral promotes N-fixation.

Pakistan is an agricultural country and the continuous use of synthetic fertilizer is causing problems for cultivable land. Excess use of synthetic 'N' fertilizers results in accumulation of NO_3^- and sometimes other organic acids in plant parts (Maga *et al.*, 1976; Venter, 1979; Kowal and Barker, 1981). Increase in disease incidences and severities had also been reported due to application of N fertilizers (Henis, 1976; Lemaire and Jouan, 1976; Krüger, 1976; Jenkyn, 1976; Temiz, 1976; Rush *et al.*, 1979). Use of these fertilizers also decreases the soil organic matter (De Bertold and Zucconi, 1980). It is a known factor that soil organic matter plays an essential role in biologically-mediated supply to plants.

Utilization of cellulosic wastes for producing biofertilizer serves two purposes, it supplies nitrogen to the soil and acts as soil conditioner. It is also a solution for the disposal of huge amounts of bagasse produced by sugarcane as industry waste. Biofertilizer also improves the soil quality and soil texture (Finstein, 1980). The purpose of present research was to utilize the bagasse for producing nitrogenous biofertilizer. In similar studies diazotrophic inoculum resulted in significant effect on crops yield (Sawfat *et al.*, 1996; Galal and Ghandour, 1996; Hegazai *et al.*, 1996).

Materials and Methods

Isolation of bacteria: Isolation of bacteria was carried out by screening seven soils and one water sample. Organisms were isolated on different selective media (Ronald, 1993). In all cases soil suspensions were prepared by mixing soil and water (1:9) and 0.5 ml of this soil suspension was inoculated in duplicate on different media. Seed cultures were maintained on Burk's medium (Rennie, 1981). Slants kept at 15°C. Stored cultures were revived at intervals of 3-4 months.

Bacteriological studies: Identification of *A. chroococcum* was carried out according to "Bergey's Manual of determinative bacteriology" (1957).

Azospirillium spp. colonies were picked up by congo red isolation method (Enrique, 1982).

All other identification tests were done according to the method described in "Handbook of Microbiology" (Morris and Maurice, 1960). Microscopic and cultural characters were also studied.

Nitrogen free broths inoculated with isolated bacteria were tested with Nesseler's reagent for the presence of ammonia or fixed nitrogen and compared with control broths (Branes, 1959).

Chemical analysis: Bagasse amended cultural broths and control were analyzed for enhancement of nitrogen content and for utilization of total soluble carbohydrates (TSC). Nitrogen estimation was carried out by semi micro Kjeldahl's digestion AOAC, (1970) followed by Nessler's reaction (Barnes, 1959). TSC was estimated by Anthrone's reagent (Chahatwal *et al.*, 1989).

Preparation of biofertilizer: Bagasse was collected from the local sugarcane juice sellers. It was initially sun-dried then oven-dried at 60-70 °C till constant weight was obtained and was crushed to small particle size 0.5-1.0 mm diameter and 3.0-8.0 mm length with the help of ball mill. Prepared bagasse was stored at room temperature in sealed plastic bags. For day-to-day experimental work bagasse samples were kept in screw cap jars.

Prepared bagasse was mixed with mineral solution (KH_2PO_4 and $MgSO_4$) and sterilized. Prepared media was inoculated with *A. chroococcum*. The resulting biomass was termed as Biofertilizer.

Evaluation of biofertilizer: Soil samples (100 gm each) were incubated separately with 20 ml biofertilizer and each sample was analyzed for the amount of nitrogen fixed or enhanced at an interval of 4 weeks upto 16 weeks.

Results and Discussion

Initially a total of seventeen microorganisms were isolated (Table 1). Out of these, four organisms could not survive on

Table 1: Preliminary characterization of isolated bacteria

S.No.	Isolate No.	Media of isolation	Gross morphology of colonies	Cellular characters	Nitrogen* fixation
1.	S2 W	Jensen	White, circular, small, slimy, 2-3 mm diameter	Very thin rods Gram-ve, motile	-ve
2.	S2 P	Nfb	Light pink, circular, small, rough, 2-3 mm	Coma-like Gram -ve, motile	+ve
3.	S3 W	Nfb	White, circular, small, 2-3 mm diameter	Coma-like Gram -ve, motile	-ve
4.	S4 W	Jensen	Same as in S2 W	Same as in S2 W	-ve
5.	S3 Y	Jensen	Light yellow, circular, small, slimy, 2-3 mm diameter	Same as in S2 W	+ve
6.	BGA-1	BGA	Round, 5-8 mm diameter, green, shining mination	Creeping filaments with fungal conta-	N.T.**
7.	N-1	*Azospirillum*	White, pin-point solid, 1-2 mm diameter	Thin rods Gram -ve motile	+ve
8.	N-2	*Azospirillum*	White, 1-2 mm diameter, solid	Same as N-1	+ve
9.	N-3	*Derxia*	Yellowish, 3-4 mm diameter, regular margin, slimy	Oval shape capsulated rods with with granules, Gram-ve, non-motile	+ve
10.	A-1	*Azotobacter*	Dull white becomes cho colate, 10-15 mm dia- meter, irregular margin, triarm cut in center, slimy, creamy	Very small dot-like Gram-ve, motile	+ve
11.	A-2	*Azotobacter*	White dark from center, concave margin, irregular hyline heap-like growth, 2-5 mm diameter, heavy slime production	Very small rods, Gram-ve, motile	+ve
12.	A-3	*Azotobacter*	Same as A-1	Same as A-1	+ve
13.	B-1	*Azotobacter*	Same as A-1	Same as A-1	+ve
14.	B-2	*Azotobacter*	Same as A-1	Same as A-1	+ve
15.	C-1	*Azospirillum*	Circular concave, white 2-8 mm diameter, trans parent shining	Curved rods with sharp ends, Gram -ve, motile	N.T.**
16.	C-2	*Azospirillum*	Same as C-1	Same as C-1	N.T.**
17.	D-1	*Azospirillum*	Same as C-1	Same as C-1	-ve

*Production of ammonia tested by Nesseler's reagent. **Not tested.

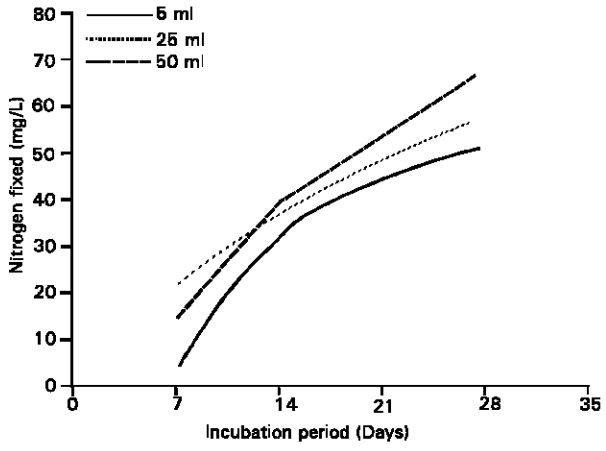

Fig. 1: Effect of inoculum size on nitrogen fixation by *Azotobacter chroococcum*

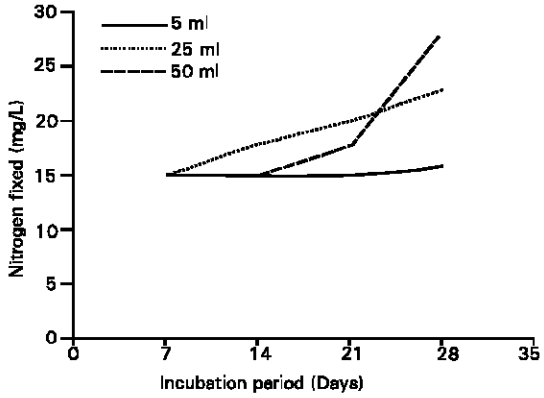

Fig. 2: Effect of inoculum size on nitrogen fixation by *Azotobacter indicus*

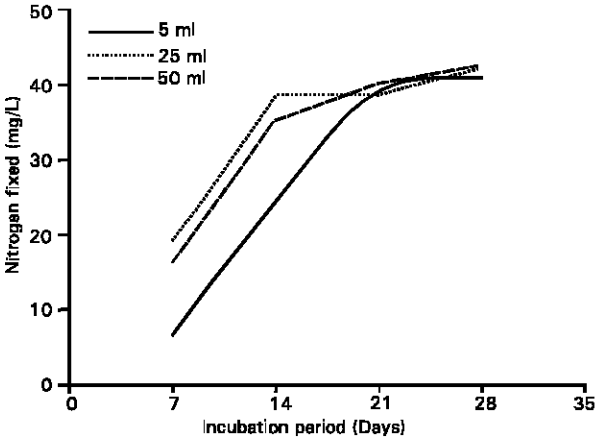

Fig. 3: Effect of inoculum size on nitrogen fixation by *Spirillum brasilense*

Table 2: Nitrogen fixation in broths inoculated by selected bacteria (15 Day).

Organisms	Nitrogen (g/L)
Non	0.00
S2P	0.05
S3Y	3.00
A-2	15.00
B-1	18.00
N-1	18.00

nitrogen-free media and fail to fix nitrogen. In remaining organisms, nitrogen fixation was tested by simple presence of ammonia in nitrogen free medium by Nessler's reagent. The cultural and microscopic studies (Table 1) revealed that among isolated nitrogen fixing organisms the characters of A-1, A-3 and B-1 were identical, while N-1 and N-2 showed similar characters and A-2, S2P, S3Y were different from these organisms. Therefore one of each group B-1, N-1 and other

Table 3: Results of sugar broths inoculated with identified bacteria.

Parameter	Organism	Days			
		7	14	21	28
Nitrogen fixed (mg/L)					
	Azotobacter chroococcum	10.00	15.00	22.00	38.00
	Azotobacter indicus	12.00	18.00	26.30	36.70
	Azosprillum brasilence	15.00	22.80	39.00	39.00
N-fixed/TSC consumed mg/g					
	Azotobacter chroococcum	50.00	50.00	57.89	76.00
	Azotobacter indicus	60.00	60.00	69.20	73.40
	Azosprillum brasilence	60.00	65.14	78.00	66.00

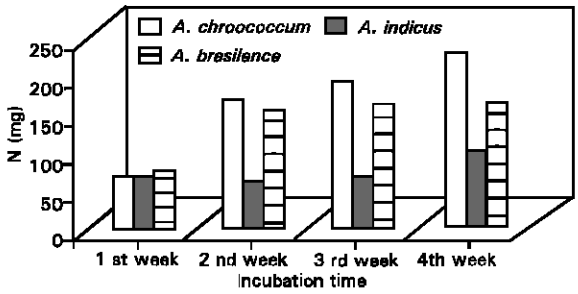

Fig. 4: Effect of 25.0 ml inoculum/l on nitrogen enhancement per gram total soluble carbohydrate (TSC) consumed by bacteria.

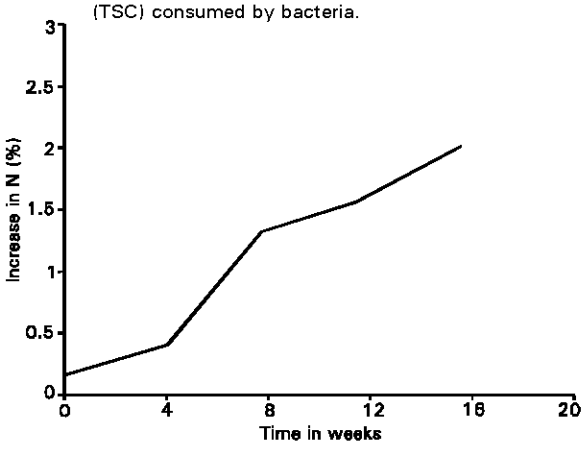

Fig. 5: Increase in nitrogen content of the soil, incubated with bagasse biolfertilizer at room temperature 35-37 °C.

organisms were examined for definite nitrogen fixing activity (Table 2). From these experimental results (Table 2) it was concluded that only A-2, B-1 and N-1 were capable to fix nitrogen in significant amount. Therefore these three were studied in detail and identified. B-1 as *Azotobacter chroococcum* A-2 and N-1 as *Azotobacter indicus* and *Azospirillum brasilense* respectively, initially these organisms were grown on sugar broth (Table 3).

The results (Table 3) revealed that in 1st, 2nd and 3rd weeks amount of nitrogen fixed by *A. chroococcum* was lower than *A. indicus* and *A. brasilense* but in 4th week the amount of nitrogen fixed was slightly higher in *A. chroococcum* than in *A. indicus* and slightly lower than *A. brasilense*.

In first week nitrogen fixation per gram TSC consumed by *A. chroococcum* was lower than *A. indicus* and *A. brasilense* (Table 3). In 1st week the values of nitrogen fixation by last two organisms were identical on the basis of sugar utilization. In second week the rate of nitrogen fixation per gram sugar consumed remained unchanged except slight increase in *A. brasilense*. In third week an increase in the amount of nitrogen per gram sugar consumed was observed in all the three organisms and the amount was the highest in *A. brasilense* and lowest in *A. indicus*. In 4th week mg/L nitrogen was maximum in *A. brasilense* followed by *A. chroococcum* and *A. indicus*. However, rate of nitrogen fixation per gram sugar used was maximum in *A. chroococcum* followed by *A. indicus* and *A. brasilense*. These results (Table 3) are in agreement with the fact that nitrogen fixation by diazotrophs require energy, which is provided in the form of soluble sugar. In nature it has been observed that growing plant exudates 10 to 15% of its photosynthates as a source of energy for rhizosphere or for nitrogen fixers (Balandreau, 1996). There might be some action of rhizosphere due to which this huge amount of energy is released by the plant. If energy is supplied artificially i.e. by addition of organic compounds to the soil it is possible to save plant's energy for better yield. When lignocellulose and cellulose breakdown products were examined (Halsall *et al.*, 1985; Halsall and Gibson, 1989a, b) as energy source for these bacteria. It has been concluded that these materials can be utilized as carbon or energy source for these bacteria.

N-fixing activity can be increased by addition of organic matter. Compounds like mannite or glucose and minerals like potassium and phosphates also increase the activity. Sundman *et al.* (1983) examined twenty compounds (polysaccharides, polyols and organic acids) as carbon and energy source for nitrogen's activity in semisolid stab cultures, of diazotrophic root associated bacteria. The studies revealed that best substrate was sucrose followed by fructose and mannitol.

From all above studies it is concluded that diazotrophs require carbohydrates as a source of energy for nitrogen fixation. The amount of nitrogen fixed increases with the increase in source of energy. Therefore, soil can be amended with lignocellulosic materials and compounds released by the action of cellulases (released by solid microbes) and can be utilized by nitrogen fixing bacteria.

In our experiments bagasse a lignocellulosic material was used and amount of nitrogen fixed in bagasse amended broths by the bacteria under study are presented in Fig. 1, 2 and 3. These experimental results revealed that in all cases amount of nitrogen fixed (mg/ml) and the rate of nitrogen fixation per gram total soluble carbohydrates (TSC) consumed increased with the increase in incubation period. The size of inoculum showed a clear effect in first week. Rate of nitrogen fixation increased with increase in inoculum size except in *A. indicus*. Maximum nitrogen fixation was observed in broths inoculated by 25 ml of bacterial seed culture for one liter bagasse amended media (Figs. 1-3). In Fig. 4, it is clear that *Azotobacter chroococcum* give the best rates of nitrogen fixation. Therefore *A. chroococcum* was selected for the preparation of biofertilizer. *A. chroococcum* was isolated locally and is familiar to our climatic conditions. Therefore it adopted to substrate very effectively. The rate of nitrogen fixation in present studies was 10 to 20 times higher than the well known stated amount that at least 100 parts carbon (organic matter decomposition) is required to bound 1 or 2 parts of nitrogen. Similar soil amendments with different

diazotrophs for maize rice and wheat have been prepared by many workers (Ahmed and Ahmedunnisa, 1984; Pigakera, 1989; Maheswari and Purushothaman, 1990; Hashem, 1996; Hegazai *et al.*, 1996; Kannaiyan *et al.*, 1996; Pedraza *et al.*, 1996; Phuong *et al.*, 1996; Pishchik *et al.*, 1996; Raicervic and Kikovic, 1996; Rashid *et al.*, 1996; Rizvi *et al.*, 1996). Our results are in agreement with these findings. Fig. 5 represents the experiment of soil incubation in laboratory. Results (Fig. 5) revealed that nitrogen fixation in soil samples was increased in 16 weeks from 0.01 to 2.0% approximately. In first 4 weeks about 0.04% nitrogen was increased. Maximum activity was recorded in next 4 weeks. In remaining eight weeks it became uniform i.e. 0.5% for each interval. Therefore, it is concluded that the biofertilizer prepared is a very good source of nitrogen as well as organic matter. Its utilization will be beneficial for our agriculture in arid and semi-arid zones.

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